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# ANAPLASMOSIS IN CATTLE



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# FOREWORD

Three important technical conferences of national scope have been held in the United States within the past ten years: one in Washington, D. C., in February 1958; a second in Stillwater, Okla., in February 1953; and the third, reported herein, in Manhatten, Kan., in June 1957. Notable progress towards means of successful control and prevention of anaplasmosis has been made in this period of time through research and the application of research findings.

The information and findings reported at the 1957 conference offer promise of a way of effective control of the disease, at least in areas where the tick is not an important vector. Antibiotics, and a workable complement fixation test, are the chief elements of a practical control program.

Much research remains to be done to further facilitate control programs for eradication of anaplasmosis. Meanwhile, research workers and others associated with the U. S. Department of Agriculture, the State Agricultural Experiment Stations, State Departments of Agriculture, and private or commercial industry are due great credit for dedicated effort and for these highly valuable contributions toward solution of the difficult problems associated with this disease.

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# THE NATURE OF ANAPLASMOSIS

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As a result of my discussions with your program committee, it is my understanding they want me to tell you about some of the facts or accumulated experimental information which we have learned about anaplasmosis. Most of the knowledge about the disease in this country has been acquired since 1927. However, from the description of the cause and transmission of so-called Texas or tick fever by Bureau of Animal Industry scientists Theobold Smith and Fred L. Kilborne, as early as 1893, we are practically certain that they were sometimes dealing with anaplasmosis associated with piroplasmosis. Later, in 1913, K. F. Meyer recognized anaplasmosis in this country from Sir Arnold Theiler's description of the disease in South Africa; and in 1926, P. B. Darlington reported on the disease for the first time in southeastern Kansas. My first contact with the disease was in the fall of 1928, when I, as a senior veterinary student, accompanied Dean Leasure to Sedan, Kansas, where we spent several days observing and studying clinical cases of anaplasmosis. During a 3-year Bureau of Animal Industry assignment in Oklahoma from 1934 to 1937, I frequently encountered and observed the disease. Since then, I have been directly associated with the Department's research group working on anaplasmosis. I should like at this time to acknowledge and give credit to the fine cooperation, assistance, and excellent teamwork of my co-workers. The information I am reporting on is an accumulation of results of work done by at least a dozen past and present members of our anaplasmosis research team, about one-half the number of whom are still active on the project. Thirty years ago Doctors Schoening and Giltner of the Pathological Division, Bureau of Animal Industry, encountered a disease of cattle in Florida which caused sickness and sometimes death. They took blood from sick Florida cattle and inoculated animals at the Bethesda, Maryland, Animal Disease Station. It was finally determined they were dealing with anaplasmosis. Continuous research on anaplasmosis has been in progress by the Bureau of Animal Industry and the newly reorganized Animal Disease and Parasite Research Division since the initial Florida disease inoculations were made.

The classification of the anaplasmosis agent has been a highly controversial subject for years. We in the Department have attempted to keep an open mind toward any evidence presented by different workers in the various sciences for support of suggested reclassification. It has been classified as being protozoan, for which it shows many typical characteristics. We believe a sound approach to this problem would be to leave it in its present classification, even if incorrectly classified, until there is conclusive evidence for its proper reclassification, because less confusion and frustration would result from its inclusion in the protozoan group than if the organism were reclassified several times in the future.

The nature of anaplasmosis can probably best be covered by reporting the characteristics of the disease in its different phases in the host, including its transmission, incubation period, parasite multiplication period, convalescence period, and carrier period.

These characteristics are as follows:

The marginal bodies, which we commonly refer to as the causative parasite, are first observed in the red blood cells as tiny specks, which on succeeding days increase in size. The number of bodies within an individual red cell may vary from one to seven, and this number is in direct proportion to the percentage of red cells which contain bodies. By careful staining and greater microscopic magnification, a number of workers are now satisfied that the large marginal bodies break apart into numerous smaller bodies. It is believed the bodies break apart at the time the red cell disintegrates. We have calculated the average life of an infected red cell containing the large marginal bodies to be 3 or 4 days. The destruction of the red cell results in an anemia. We can determine quite accurately what the red cell count or the degree of anemia will be 3 days later by checking the percentage of red cells affected.

The marginal parasite is located inside the red cell. It is very fragile and does not retain its infectious character except for short periods outside of the animal body. Its survival outside of the red cell is apparently of still shorter duration. It also is very sensitive to changes in the environmental fluid surrounding the infected red cell. For example, repeated experiments with infected cells from an acute case, when diluted in physiological saline, lost their infectivity after 30 minutes.

The infectious agent from acute cases will survive in undiluted citrated or defibrinated infected blood held at room temperature for only a few days. It may survive for a month or less at ordinary refrigerating temperatures (40° F.), and we have had several successful transmissions with frozen red blood cells held in a dry-ice storage chest from a few hours to 8 years. We hoped to preserve different experimental field strains in the frozen state rather than to maintain carrier animals for a source of seed material, but this method could not be relied upon as too many frozen samples failed to survive in storage.

One freezing and thawing of infected blood cells will lyse most of the red cells, and, correspondingly, will destroy 95 percent or more of the infectious agent. Infectivity is totally destroyed by more than one freezing and thawing. Between 50 and 60 animals were inoculated with large volumes of blood and tissues from acute cases which had been frozen and thawed several times without infecting a single animal.

Recently we have learned the agent is very sensitive to phenol. Normal serum containing 0.5 percent phenol mixed with freshly drawn infected carrier blood and allowed to stand at room temperature for 2 hours destroyed the infectious

agent, but a control sample treated with the same nonphenolized serum infected a nonsplenectomized cow. Other chemicals, such as the antibiotics, oxytetracycline, and chlorotetracycline, have proved to be destructive for the agent in the carrier animal.

Transmission. The natural transmission of anaplasmosis needs much more intensive study even though we think we know much about it. Intrauterine transmission from the infected dam to her offspring was proved in a few cases by our early workers, but our present serological evidence indicates this type of infection occurs infrequently. We believe, and are now attempting to prove experimentally, that this type of transmission usually takes place in the pregnant dam during the acute course of the disease, at which time the infectivity level of the dam's blood would contain as much as a hundred thousand to a million times more of the infectious agent than when she becomes a carrier.

The disease is not spread by contact, but it can be spread from an infected to a susceptible animal by biting or sucking insects, bleeding needles, vaccination needles, tattooing needles, or castration or dehorning instruments; or, theoretically, it can be transmitted mechanically by anything that will carry infected red blood cells from one animal to another. The problem of mechanical transmission is important, but it can be controlled. We believe our present knowledge of the fragility of the parasite, infectivity blood levels which correlate with antibody levels that can be measured by the complement-fixation test, and information which we have developed on the large amount of blood from low-titer-reacting carriers needed to infect susceptible animals offers new possibilies in practical control and eradication of this disease.

For example, 50 ml. of blood collected from each of 20 suspect reacting of animals in an infected herd when inoculated into susceptible splenectomized calves resulted in negative reactions, whereas 500 ml. of blood collected from four similar suspects in the same herd and inoculated into splenectomized calves resulted in four acute cases of anaplasmosis. Our interpretation of these results is that such suspects are of no immediate danger among negative animals because it would by physically impossible for insects to transmit that volume of infected blood.

Our research group and probably most other workers have previously considered the inoculation test as being practically 100 percent accurate, for which reason it has been used as the measure for evaluating the accuracy of the complement-fixation and other tests. The results obtained with the inoculation tests on the previously mentioned suspect reactors, as well as with some of our other inoculation studies, indicate that the accuracy of the inoculation test may be much less than 100 percent, and it appears less accurate than the complement-fixation test. Its accuracy will also depend on the volume of blood inoculated, the time interval between collection and inoculation of the blood (which should not exceed 24 hours), the temperature at which the blood is held or shipped, and the susceptibility of the test animals.

Blood shipments for inoculation should never be frozen, nor should they be exposed to heat or fluctuating temperatures. We have recommended icing of blood shipments with frozen cans of water to prevent multiplication of bacterial contaminations and to maintain a more constand temperature, even though we know

there is a marked loss of infectivity titer following overnight storage in a refrigerator. We believe the loss of infectivity titer is decreased by holding the blood at room temperature, 70° F., provided temperature fluctuations do not occur.

Our criteria for a susceptible test animal is a calf which has been splenectomized 4 to 6 weeks prior to inoculation. Stained blood smears are examined microscopically, and serum samples are tested by the complement-fixation test before splenectomy and three times a week after splenectomy. This procedure will allow for the detection of blood infections, such as hemobartonellosis, eperythrozoonosis, theileriasis, and anaplasmosis, which are known to interfere with inoculation results. The 4-to-6 week time interval also usually allows sufficient time for hemobartonellosis, eperythrozoonosis, and theileriasis reactors to recover from acute infections which flare up following splenectomy. Animals recovered from these acute infections have been found to be susceptible to anaplasmosis without any recognized interference.

Calves which are negative to an inoculation should not be used again for test purposes because some of these animals develop nonclinical anaplasmosis and immunity. Some other negative-test calves develop a resistance to inoculations, the cause of which we have not yet been able to explain. Negative-test calves should be challenged with a lethal dosage of parasitized red blood cells to prove their susceptibility. For challenge purposes we use 0.5 ml. per pound of packed red cells which contain 50 percent or more marginal bodies.

The transmission of anaplasmosis by the tick is reported to be an entirely different problem from mechanical transmission because the infectious agent will survive and possibly multiply in the tick as transovarian passage of the infectious agent has been proved in certain species of tick. We don't know of anyone who has the answers to the tick transmission problem, but we now have two anaplasmosis cooperative projects on ticks with the Department's Entomology Research Division, from which studies we should learn something in the next year or two. We have been interested in and very much encouraged with the results of a field trial study by Doctors H. Marsh and E. A. Tunicliff on a herd of cattle at the North Montana Experiment Station, where they appear to have been successful in eradicating anaplasmosis by testing and slaughter or treatment in an area populated with the Rocky Mountain spotted fever tick, _Dermacenter andersoni_, which is suspected of being one of the most common vectors.

_Incubation Period._ Our definition for the anaplasmosis incubation period is the interval between the time of exposure and the time when the first marginal bodies can be detected in the stained blood smears. The average incubation in experimental animals receiving carrier blood or small dosages of acute blood is between 14 and 45 days. We should expect the incubation period for natural field cases to be of similar duration or longer. However, the longest experimental incubation period we have ever observed was 60 days. Recently we thought we had an incubation period of 105 days, but we could not transmit the disease from that animal.

The incubation period is one phase of this disease which we do not at this time completely understand and for which we cannot offer a proved explanation about the location of the infectious parasite nor determine what it is doing. We suspect the organism is passing through a portion of its life cycle which is necessary for its existence in the host, because a similar cycle has been observed in the carrier animals on numerous occasions following recovery from acute infection. Another reason we suspect the organism is passing through a vital portion of its existence during the incubation period is that on numerous occasions we have observed evidence that the organism was attempting to establish itself in the susceptible animal, in which marginal bodies failed to appear. For example, one such animal's blood, when passaged to another susceptible animal, produced an acute case. Some other similar animals receiving small titration dosages of known infectious material developed a slight anemia at the exact time they should have done so if marginal bodies had been present. Serial bleedings of serum from some inoculated test animals have shown a suspicious complement-fixation reaction for a few days at about the correct time for the initial reaction, which was followed by negative serological reactions and a number of negative-test animals have shown either complete or partial resistance to a 60-day postinoculation lethal challenge exposure.

We can experimentally control the length of an incubation period or entirely eliminate it by regulating the inoculation dosage of parasitized red blood cells. For example, susceptible animals receiving 0.5 ml. of packed red cells containing 50 percent marginal bodies per pound of body weight will not go through an incubation period. We believe that the parasite is located in the red cells during the incubation period at the time of the theoretical vital life cycle phase because the carrier animal's red cells, containing no visible marginal bodies, do contain the infectious agent, and we can here again affect the length of the incubation period by increasing or decreasing the volume of the inoculum.

<ins>The Parasite Multiplication Period</ins>. The parasite multiplication period in the acute case is the period of time following an incubation period when the marginal bodies appear in the red cells. Following their initial appearance the number of red cells showing marginal bodies will double approximately every 24 hours for 7 to 11 days, after which time either the animal will die from an anemia or the percentage of parasitized red cells will gradually decrease as they are replaced by new red cells. We have learned that the parasite multiplication rate is a fairly well fixed characteristic of any particular field strain, but different field strains may show considerable variation in this peculiar phenomenon. It is of considerable interest that we worked for about 10 years on experimental antigen production for the complement-fixation test, during which time we used only the old, original Florida strain previously mentioned. We made hundreds of animal passages during that time with the primary objective of improving our red cell parasite counts, because we knew that would improve our antigen. During all those passages we were never able to alter or change that parasite multiplication rate, so we concluded that was a fixed characteristic of <ins>Anaplasma marginale</ins>. Following this experience our Florida production strain became so heavily contaminated with <ins>Hemobartonella</ins> that we were forced to discontinue its use and screen several other available strains for antigenic properties.

As a result of this screening, we were amazed to learn there was a marked difference in the parasite multiplication rate for some of the different field strains. We found one field strain from Virginia which showed such a rapid parasite multiplication rate that blood could be harvested in from 4 to 6 days, and we found another field strain from Delaware which showed such a slow parasite multiplication rate that blood could not be harvested until about the 10th or 11th day. The ideal situation for antigen production would be to have all the red cells become invaded by marginal parasites at one time so there would be only a slight loss of disintegrated cells. We usually lose 50 percent of the red cells in most animals from the resulting anemia before we can get the parasite count sufficiently high for harvesting.

In addition to those antigenic differences of different field strains, several other foreign and some domestic workers have from time to time reported a marked difference in the virulence and pathogenicity of some field strains of anaplasmosis. For example, the first observed and reported difference in the virulence of different strains of anaplasmosis was published by the South African workers as they isolated a strain which they named Anaplasma centrale, which produces a milder form of the clinical disease in cattle and gives immunity against the more virulent A. marginale. About 10 years ago we had a visit from a veterinary officer from the British Colonial Office, who was assigned to Jamaica to assist the local government in reducing the heavy losses from animal diseases. He reported anaplasmosis was a major problem. He stated the local field strain was so highly virulent that they were losing most of their young weaned dairy calves soon after they were moved from screened barns to an outside environment. He said they were also losing most of their imported breeding cattle from Canada and other British colonies.

More recently, Dr. T. E. Franklin at College Station, Texas, informed us he had a virulent field strain which would kill some inoculated nonsplenectomized calves. He forwarded to us some infected blood, which we inoculated into two normal nonsplenectomized calves. Our results confirmed his report concerning the strain virulence, as the disease was the most severe we had ever observed in normal calves. It should probably be mentioned that, ordinarily, nonsplenectomized calves show a market resistance to anaplasmosis and natural clinical cases are very seldom observed in the field.

There is another interesting and probably very important phenomenon associated with the parasite multiplication period, which is the development and accumulation of a highly toxic agent in the blood from acute cases of the disease. This toxic agent appears in approximately equal concentration in the serum and the washed red cells. It seems to reach its maximum concentration at about the same time as, or shortly following, the maximum height of the parasite count. We used to lose many of our inoculated animals that had been given large dosages of infectious whole blood. This was very discouraging, because it meant we had lost our passage series and we should have to start a new passage series from a carrier. The inoculated animal that had been given the toxic material would usually show signs of shock within an hour, and some animals would show clinical symptoms before the inoculation needle

could be removed from the jugular vein. The most common symptoms were bloating, rapid respiration, diarrhea, accelerated pulse, and, frequently, hemorrhage from the nostrils and rectum. These animals would respond to treatment with intramuscular injections of epinephrine hydrochloride, but it was necessary to observe them every hour or two for 24 hours, because recurrence of the clinical shock symptoms would sometimes develop.

We overcame the problem in our work by removing the plasma from the red cells, which were given one wash in physiological saline before being inoculated.

We believe that the toxic agent is either a protein decomposition product resulting from the destroyed red blood cells or a toxin produced by the anaplasmosis parasite. It is possible that this toxin or protein decomposition material could be the cause of death in some peracute cases of the disease when death occurs before a severe anemia has time to develop. It is also of interest that our difficulties with anticomplementary antigens were associated with the same late blood-harvest period, when the toxic agent is in its greatest concentration in the blood.

Some protein analytical studies are needed in this field of work.

The Convalescence Period. The convalescence, or recovery, period starts with the decline in the number of parasitized red cells and usually lasts for 1 or 2 months, depending on the severity of the acute reaction, the rate of reproduction of new red cells, and the presence or absence of secondary complications. Animals showing a red cell volume of 10 percent or less are in extreme danger and should receive an immediate transfusion of citrated bovine blood. The prognosis of most acute cases may be determined by an examination of the stained blood smears for the presence or absence of new red cells. Numerous new red cells on the slide of any animal's blood is a good indication that animal will recover. Young animals show a much greater response to the need for new red cells than older animals, and consequently most death losses from an anemia occur in the older animals.

The first attack of marginal bodies in an animal is always the most severe; then, frequently, there is a less severe second new invasion of some red cells again after 2 or 3 weeks. Some animals will have several irregular periodic mild red cell invasions even up to a year or longer after the initial attack. As previously stated, we associate these recurrences of red cell invasions with a parasite cycle development similar to malaria.

The Carrier Phase. We describe the carrier phase of the disease as the period after visible marginal bodies have disappeared from the red cells. Most animals that show an acute infection will remain carriers of the disease for life, but there have been some known cases which lost their carrier infection and again became susceptible to reinfection. We believe, as a result of a few limited experimental tests, that in nature there may be many mild, nonclinical cases of anaplasmosis which occur when the animal is given a light exposure. It is also probable

that many more of this type of carrier lose their carrier state than do carriers which had severe clinical reactions. We have definite proof that the infectivity titer of the blood is directly correlated with the number of marginal bodies in the red cells. It has also been proved that the infectivity titer ascends with, and gradually drops along with, the serum antibody titer after the initial disease attack.

We are attempting to make infectivity titrations in susceptible splenectomized calves from many different infected acute and carrier animals, each of which has different levels of complement-fixing antibody, so as to establish the correlation of infection and antibody at all levels. This information not only will give the research worker a valuable new tool in the complement-fixation test but it can also be used by control officials to pick out the dangerous high-titer reactors for isolation or elimination from infected herds, and thus probably prevent outbreaks of acute cases where it is not practicable for an owner to test and slaughter reactors or segregate reactors from negative animals. The use of such a plan needs to be tried on a few herds in different areas of the country to determine its value in preventing the spread of the disease by vectors. The morbidity and mortality losses from acute anaplasmosis is well recognized, but the effect of carrier infection on the thriftiness, growth, milk production, weight gains, and general condition of dairy and beef cattle needs to be studied. It is highly probable that the hidden losses from carrier infection may far exceed the more spectacular losses from death and sickness. Dr. E. H. Willers, from Hawaii, has stated that different Hawaiian dairymen have reported that the removal of reactors from their dairy herds also removed the low milk producers. Carrier infection could be a contributing factor to the comparatively lower milk production from southern cattle than from northern anaplasmosis-free cattle. This is also true of experimental dairy herds of the same blood lines.

Immunity. There appears to be only one immunological type of anaplasmosis of cattle and one immunological type of the disease in sheep, neither of which will cross-infect or cross-immunize the opposite species.

The immunity level of an infected animal corresponds with the level of infection, and a loss of carrier infection is followed shortly by a loss of any immunity which that animal had, making it again susceptible to reinfection.

Carrier animals will not usually show any marginal bodies in their red cells following inoculation with small dosages of infectious material, but large doses of acute blood, which is lethal for susceptible cattle, will usually cause the development of marginal bodies in from 1 to 10 percent of its red cells.

Some individual Brahman and Brahman crossbred cattle show a natural resistance or immunity to challenge, whereas other Brahmans, including both purebreds and crossbreds, appear as susceptible to challenge as our American dairy and beef breeds.

Vaccination of susceptible cattle with killed anaplasmosis antigens resulted in the animals' showing positive reactions to the complement-fixation test, and many animals developed sufficient immunity to prevent clinical cases of the disease following challenge. However, all challenged animals were found to be carriers when their blood was inoculated into splenectomized calves.

The serum from several calves 3 days to 4 weeks of age, born from infected dams, gave positive complement-fixation reactions, but a retest on these same animals at a later date showed all sera were negative to the test, indicating there was a transfer of antibody without infection in the colostrum or milk from the dam to its offspring.

I should like to summarize my report by stating that I feel the accumulation of our knowledge about anaplasmosis equals or exceeds the information which was available to the disease-control officials for those diseases which have been or are in the process of being eradicated. Some of those diseases are bovine contagious pleuropneumonia, piroplasmosis of cattle, foot-and-mouth disease, tuberculosis, brucellosis, and, more recently, vesicular exanthema of swine.

We feel that the nature of this disease is such that it could probably be successfully eradicated from tick-free areas without much difficulty and that it may even be possible to eradicate it from tick-infested areas if we try. We should probably need to develop tick-control programs and eliminate the high-titer-reactor-infected cattle so as to dilute down the infection which supplies the tick-reservoir infection on pastures and other land. Our present greatest need is for your assistance and cooperation in developing information which will help those livestock people in areas where the incidence of both anaplasmosis and ticks is high.

# DISTRIBUTION AND INCIDENCE OF ANAPLASMOSIS

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Anaplasmosis has been reported as having been diagnosed in 38 of the 48 states. However, many of the north central states should not be included in the anaplasmosis infected area of our country as most of their reported cases have been traced directly to animals imported from areas where the disease is considered endemic.

For a good many years, it was thought that anaplasmosis was limited to the original tick fever states and probably was contained in those states for a long time by the quarantine lines established to control tick fever. With the development of modern transportation, many exposed and recovered carrier animals have been moved into previously anaplasmosis clean areas and the disease has become established in many herds. This condition is not classed as a reportable disease in many states as yet and in others, only recently; making it difficult to compile anything like an accurate report of the distribution and incidence of anaplasmosis in the United States.

It would appear that the anaplasmosis line, if it may be called that, has advanced from the old fever tick line in the South to roughly one extending along the Maryland-Pennsylvania boundry westward to the Ohio River, following that to the Mississippi River, up the Mississippi to the Missouri-Iowa boundry, westward to the western boundries of Nebraska and the Dakotas. Anaplasmosis can be expected to be found in the area south and west of that line. The true incidence is not known except from some survey work that has been done in certain areas, since the development of the complement-fixation test to determine the carrier animal.

It has been generally recognized for years that a belt, where the disease is endemic, extended from the tidewater area of Maryland down the Atlantic coast to Florida, around the coast to the Mexican border. The boundries of this belt island vary according to terrain and the vector population responsible for the spread of the disease. The only area in this belt in which any appreciable survey work, using the complement-fixation test, has been done, with which I am acquainted, is in Maryland. Dr. E. J. Poelma, College Park, Maryland, reports a high incidence of herd and animal infection in the eastern part of the state but found that it was reduced west of the Chesapeake Bay until only occasional animal infection was disclosed in the western part of the state. At some time or other anaplasmosis has been disclosed in most counties of the state, but the cases found in the western counties nearly always have the history of being moved from the tidewater counties. The exiguous testing accomplished in Virginia indicates a similar situation in that state. It seems fair to assume that this would apply on around the coast.

The animal disease reporting systems of the states that list anaplasmosis as a reportable disease were utilized to plot the cases reported by counties to determine the distribution. It is recognized that this does not give a true picture of the distribution, let alone the incidence, as this information is restricted to the clinical cases observed and reported by practicing veterinarians, for the most part. However, by adding this information to the knowledge gained over the years by many who have reported on this disease, and applying the results of the complement-fixation testing that has been reported, we are able to indicate with anticipation of a fair degree of accuracy the relative degree of incidence. The map we have developed reflects this. For example, "Slight incidence" is a relative term. . . . it is difficult to indicate occasional cases that are reported in western Kansas, Oklahoma, and Texas. However, we should not be surprised to have it diagnosed there.

There are areas on our map within the boundries of the anaplasmosis area previously outlined that could be interpreted to mean that no anaplasmosis exists. If a complete test of all the cattle in those areas was accomplished, I feel there would be carrier animals disclosed that would be classed as positive to the test. More complement-fixation testing for anaplasmosis needs to be done so that the true incidence can be determined.

Most of you are aware of the recent report by Splitter, Anthony, and Twiehaus on their survey of the incidence and distribution of anaplasmosis in Kansas. Their work indicates that it is primarily confined to the eastern one-third of the State, with 52 out of 56 reactors and suspects from 5,000 animals tested, found in that region.

The anaplasmosis complement-fixation testing that has been done in Oklahoma, while not done as a statewide survey, and other reports of the disease indicate that it is greater in the southeastern part, and fairly well restricted to the eastern third of that state also.

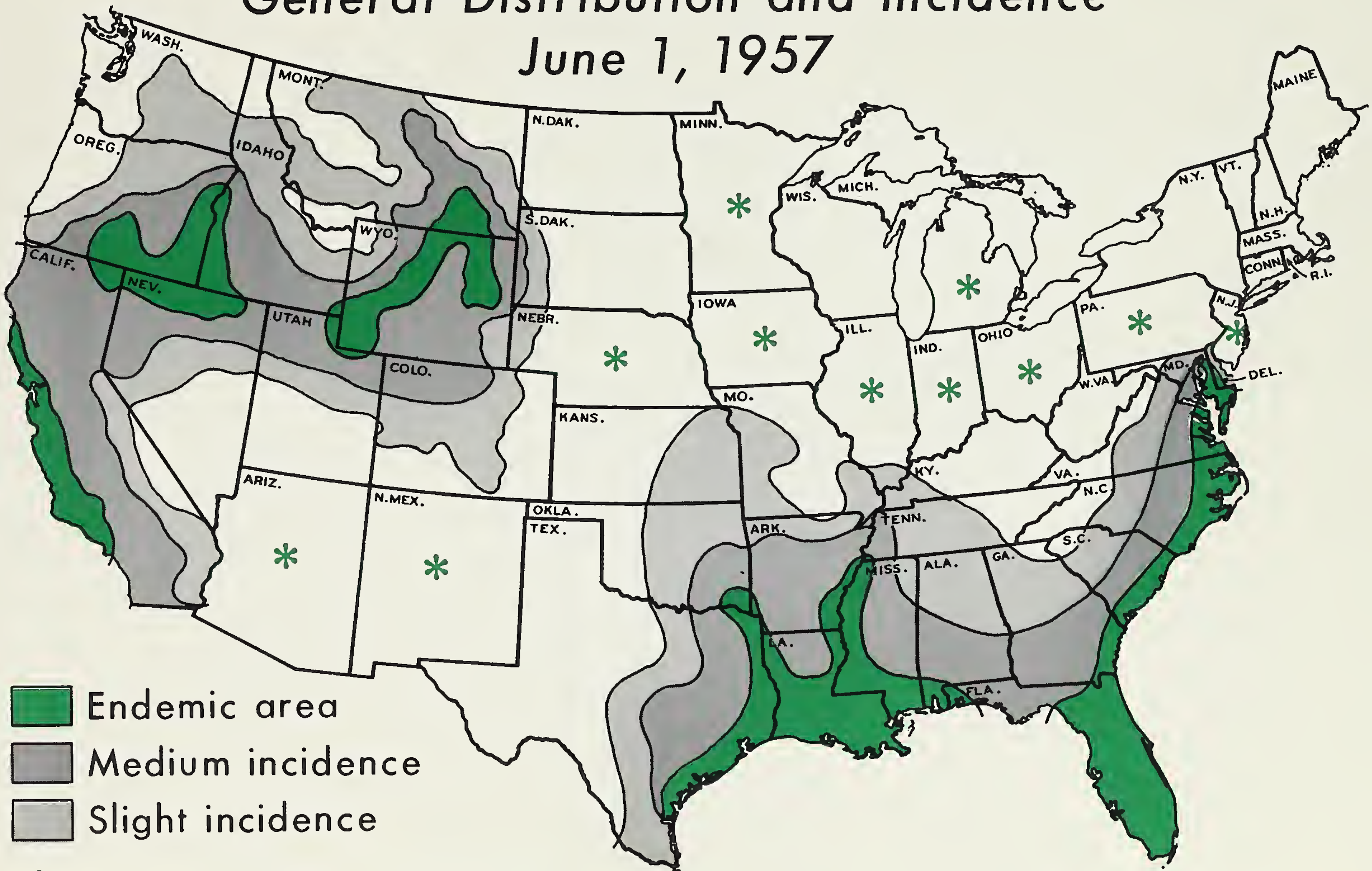
One of the more comprehensive surveys by means of the complement-fixation test has recently been completed in Wyoming. This work has not as yet been published, but George M. Thomas, ADE Division Serologist at the cooperative brucellosis laboratory, has conducted tests on 6,700 cattle from 20 of the 23 counties representing 388 herds. The blood serum samples were selected at random from samples submitted for brucellosis tests from both beef and dairy herds. Of the 6,700 sera tested, 702 or 10.48 percent were classed as reactors and 176 or 2.62 were designated suspects for a total of 13.1 percent. Infection was disclosed in 18 of the 20 counties sampled.

Anaplasmosis has been diagnosed in 24 of Montana's 56 counties with the greatest concentration in the south central part of the State.

In March of 1955 a survey was conducted in Malheur County in Oregon. Twenty five herds comprising 2362 head were C/F tested with 1314 classified as reactors and 91 suspects for a percentage of 59.4 carrier animals. It is also

# ANAPLASMOSIS

## General Distribution and Incidence
## June 1, 1957

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diagnosed in the western or coastal region of the State but usually the movement of cattle from the endemic areas are involved.

Anaplasmosis has been diagnosed in about all counties in California but no survey work to determine the incidence has been conducted. Some herd testing has been done which would indicate the greater concentration to be along the coastal counties. Of the 5737 head tested for export to Hawaii during the past two and one-half year, 378 or 6 58 percent have been classified as reactors or suspects.

In developing the map to indicate the distribution, and by shadings to imply somewhat the incidence, we were unable to indicate where the occasional case might be reported. For example, Arizona and New Mexico have encountered the disease but it is not considered to be a problem in those States, and it has been suggested that cattle imported from other States have been involved in the cases diagnosed. This is true also of the States that are indicated to be outside the anaplasmosis area, but are starred to indicate that the disease has at some time been diagnosed within their boundries.

It is my belief that the disease is much more widespread, of a greater incidence, and extracts a greater toll from our cattle industry than is generally recognized. To determine this more accurately, it is necessary to train more people in the technique of the anaplasmosis C/F test and increase the testing facilities. The Animal Disease and Parasite Research Division of the Agricultural Research Service has heretofore produced the antigen being used by the Animal Disease Eradication Division We are now attempting to make arrangements for antigen production so that survey and diagnostic testing can be continued and expanded. Until this production is assured, we will, of necessity, have to be cautious of further commitments for its use.

# TRANSMISSION OF ANAPLASMOSIS BY ARTHROPODS

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The first recorded demonstration of the transmission of anaplasmosis by arthropods occurred when Smith and Kilborne placed infected fever ticks, Boophilus annulatus, on non-immune cattle. They were studying the transmission of tick fever and considered the small "peripheral coccus-like" bodies which appeared in some animals, some time after piroplasmosis developed, as a secondary manifestation of Piroplasma bigeminum. Their contribution to the knowledge of piroplasmosis was quickly recognized, but it was 40 years before Dikmans clearly demonstrated that they had transmitted both anaplasmosis and piroplasmosis by Boophilus annulatus.

In 1912 Thieler showed that Boophilus decoloratus and Rhipicephalus simus were able to spread anaplasmosis. Many workers have continued transmission studies, until to date 19 species of ticks belonging to seven genera have been incriminated.

It soon became evident that in some parts of the world tick transmission was not the most important mechanism of spread. Anaplasmosis outbreaks occurred in parts of the United States where ticks are almost unknown. In 1930 Sanborn et al. at the Oklahoma Agricultural Experiment Station demonstrated that horseflies were effective vectors of anaplasmosis. At least nine species of horseflies have now been incriminated by several workers. Howell and co-workers showed that mosquitoes may be vectors under some circumstances, but they concluded that these insects were seldom important factors in the spread of anaplasmosis. Stableflies and hornflies have been suggested as vectors, on epidemiological grounds; but to date they have not been clearly incriminated experimentally.

## Mechanism of Transmission

Two distinct types of transmission of anaplasmosis by arthropods have been demonstrated and an understanding of the problems involved is necessary in planning large scale control measures. Some arthropods serve exclusively as mechanical vectors, while others may be biological vectors. In a few cases ticks may serve as both biological and mechanical vectors.

## Mechanical Transmission

Mechanical transmission is the introduction of Anaplasma organisms from infected to normal animals by means of contaminated mouthparts in the same manner as contaminated instruments may transfer the disease organism.

The mouthparts of horseflies are admirably adapted to accomplish this act. They are composed of six efficient, rapier-like cutting structures which penetrate

the hide of animals until a blood supply is reached. Then a powerful anti-coagulant is introduced to keep the blood fluid until it can be picked up by the sponge-like labellum. Should the fly be disturbed before its blood meal is completed a new wound will be made on the same or another animal, and the contaminated mouthparts may introduce the Anaplasma organism.

A rough correlation may be shown between the size of the horsefly and its ability to spread anaplasmosis. Of even greater importance are the feeding habits of the individual species. The big black horsefly Tabanus atratus is one of our largest and most numerous flies, yet it is a relatively poor vector. It is easily disturbed during feeding and seldom resumes feeding, under experimental conditions, in less than an hour. In contrast, T. sulcifrons, a slightly smaller fly, is not easily driven away. When disturbed during feeding it flies about for a brief interval and resumes feeding. T. abactor, a smaller species, has similar habits and despite its smaller size is considered one of the principal vectors where it is numerous.

The possibilities of transmissions by deerflies have been studied extensively. They are not easily driven away but, presumably because of their small size, only a small number of transmission experiments involving deerflies have been successful.

Mosquitos are proven mechanical vectors but probably seldom greatly influence the amount of anaplasmosis in an area. Their mouthparts are relatively small and they lack the large, sponge-like labellum of the horseflies which serves as such an efficient method of transferring blood from one wound to another.

The interval between the infective and infecting feeding is very important in mechanical transmission. No case is recorded in which over five minutes have elapsed between the two feedings. This factor is important in determining the probability of transmission of anaplasmosis to other herds by arthropods. Spread of more than a mile is very unlikely if mechanical vectors only are concerned.

## Biological Transmission

Transmission of anaplasmosis by arthropods which occurs long after the infection was picked up by the vector is called biological transmission. It has been demonstrated only in ticks and may occur from several days to several years after the ticks become infected as a result of feeding on sick or carrier animals. Occassionally the disease may be spread before the tick molts again, but more frequently the Anaplasma organism is transferred following one or more ecdyses. The infection may be picked up in the larval stage and transferred to a normal animal by feeding during the nymphal stage, or infection may not occur until the tick feeds during the adult stage. Under very favorable conditions the organism may be picked up in the larval stage and transferred to the nymph, adult, next generation larva, nymph and adult and still be capable of infecting non-immune cattle. This may require at least six years.

The ability of ticks to carry *Anaplasma* organisms for years is very important in disease control procedures. Tick infested pastures where anaplasmosis has occurred cannot be considered free of the disease for at least six years. In addition, many of the tick vectors can survive on many hosts, so pasture rotation cannot be relied upon to eradicate ticks as it was with the cattle fever tick. Tests with *Amblyomma americanum* indicated that no more than 85 percent control of ticks could be obtained by keeping the pastures free of cattle for 10 years. When extensive trapping and shooting of all known hosts of *Amblyomma americanum* was practiced in the cattle-free area, over 95 percent control occurred.

The ease and speed with which ticks infested with anaplasmosis may be carried long distances by their hosts creates another serious problem. Birds or mammals may carry infested ticks across state lines or from infested to non-infested areas very rapidly, suggesting that any eradication program should be very extensive in scope or limited to areas with very effective natural barriers such as the Hawaiian Islands.

## Summary

Anaplasmosis may be spread by arthropods either mechanically or biologically. Horseflies and mosquitoes are proven mechanical vectors, and many other bloodsucking insects such as stableflies and hornflies are probable vectors. Mechanical transmission is possible for short periods only, probably seldom more than five minutes. This greatly limits the distance the disease can be spread mechanically. On the other hand, ticks, the only known biological vectors, may spread the disease for years and during this period may be carried hundreds or thousands of miles. Control and eradication procedures must take into account the arthropod vectors if satisfactory results are to be obtained.

# THE ACCIDENTAL TRANSMISSION OF ANAPLASMOSIS

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The subject assigned to the speaker is "The Accidental Transmission of Anaplasmosis." Accidental transmission would be that which is carried other than by natural vectors, not including deliberate transmission. Then deliberate transmission is that which is done in attempt to immunize young animals by the injection of non-carrier blood.

Any transfer of blood from a carrier animal to a susceptible animal could be capable of producing the disease in the susceptible individual. Such operations as dehorning, castration, ear notching or other surgery should be conducted in a manner which would eliminate any possibility of carrying the disease from one animal to another. Further, such simple operations as vaccination or immunizations against such common diseases as blackleg, shipping fever, brucellosis, and collecting blood samples, all could be means of transmitting the disease, as blood could be carried from one animal to another on a needle or instrument. It has been my observation that in official work one of the most common procedures is overlooked--that is, the use of the tattoo instrument. This instrument should be disinfected between each operation, the same as any other surgical instrument, to prevent the possibility of transmitting this or any other disease. It is imperative that the veterinarian or others disinfect his instruments between each operation whether it be a set of surgical instruments, the dehorning shears, knives, saws, hypodermic needles, ear notchers or tattoo instrument, just the same as though major surgery is involved. Such disinfection can be done by chemical means after adequate cleansing, and I am sure everyone can work out a very simple procedure that would be adequate to disinfect and prevent the possibility of tracking the infection.

The practice of jabbing animals with a stick, prod, nail, or other sharp point is another means of transmitting the disease; however, this practice has been pretty well discontinued in this country. Older animals being worked are more apt to accidentally transmit anaplasmosis; the older animals have had a better opportunity of contracting the disease and being in the carrier state.

The possibility of transferring carrier animals from one locale to another has been greatly increased because of our marketing practices, including our numerous livestock auction markets and our modern high speed transportation. As man travels so do his animals and so does disease; and certainly we in this nation are on wheels and we are moving our animals further and more frequently than ever before. There appears to be a greater turnover of animals on farms and ranches than previous, therefore, this should increase the possibility of introducing infected or carrier animals.

No figures have come to my attention as to the estimated accidental transmission of the disease. However, there is the possibility, and it must be guarded against.

We, as veterinarians, should certainly conduct ourselves in our procedures at all times in such a manner that there will be no possibility of our being the disseminator of any infection; whether it be sterilizing hypodermic needles or washing our boots. We are endeavoring to prevent diseases, as well as bring about a recovery when they occur. Certainly here is a place where an ounce of prevention is worth many pounds of cure. Since being in the regulatory field it has come to my attention that it does us all good to review procedures, especially in this field. If we can go over the "Why" we do these things, it is much easier to not become lax but conduct our work in a manner above reproach; then, if we are unfortunate enough to have an outbreak following dehorning or immunization for brucellosis or other we know that we have as far as possible not been a contributing factor.

In serving the public it is a part of our responsibility to give them a better understanding that it is necessary to take such precautionary measures since many of them may do some of these procedures themselves. It has been the observation of your speaker that an informed client is your best client.

# THE CLINICAL DIAGNOSIS OF ANAPLASMOSIS

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My subject is the clinical diagnosis of anaplasmosis.

I have given considerable thought to my approach and treatment of this subject. However, after receiving the conference program a couple of weeks ago and noting how thoroughly the disease was to be reviewed by the many speakers, I felt my best contribution would be to simply relate the experiences of a veterinary practitioner with the disease.

I have had the opportunity to constantly observe anaplasmosis on the beef cattle ranges in Santa Barbara County in California for the past ten years.

This area has considerable mountain and foothill country whose sole use is to pasture range beef cattle. This range country is covered to a large extent with sage-brush. This brush is inhabited by millions of ticks and they are all, apparently, solidly infected with *Anaplasma marginale*.

A good amount of the beef cattle industry is seasonal. Rains begin about Thanksgiving and terminate about May 15th. Probably 40 or 50 thousand cattle are imported each fall to pasture the resulting green grass, and then are shipped to feed lots about June 15th when the grass has died due to lack of moisture. These imported cattle come from the west, the northwest, the southwest, and even from many of our southeastern states. Many have not been previously exposed to anaplasmosis and the result is, at times, a considerable incidence of anaplasmosis in our area. Many local cattlemen have become intimately familiar with the disease. As a result, in recent years, we have observed a preponderence of young weaner calves being imported in order to avoid the acute and fatal form of the disease.

The clinical symptoms of anaplasmosis can be classified, as in many diseases, as being mild, acute, and peracute. However, there are, first, five cardinal symptoms which when found together usually spell anaplasmosis and only anaplasmosis. They are:

1. anemia
2. weakness
3. a febrile reaction
4. grossly normal urine
5. constipation

Secondly, we usually find the following:

1. icterus
2. inappetence
3. depression
4. dehydration

5. labored respiration
6. irrational behavior

Thirdly, we must note that, clinically, it is a disease which is generally mild in calves up to one year of age; acute but not often fatal in cattle up to two years of age; acute and often fatal in cattle up to three years of age; and often peracute and usually fatal in cattle over three years of age. Fourthly, it is clinically important to note that no weak and very anemic animal can tolerate physical exertion. Treatment requires at least some restraint. Therefore the prognosis is always much more unfavorable in a wild range animal than in the so-called farm animal accustomed to being handled by man.

Differential diagnosis of anaplasmosis is usually not difficult. It is readily differentiated by laboratory means. Clinically, although it manifests the anemia, weakness, and icterus of many of the contagious bovine anemias, it can be rather accurately diagnosed by the grossly normal urine and the presence of constipation no matter how green and lush the forage. The relative age of the animal is also important clinically. In the bovine anemias found in our practice other than anaplasmosis, the acute cases are found in the younger animals while the older group manifest either mild or subclinical symptoms.

On post mortem in anaplasmosis, anemia and/ or icterus are evident, the kidneys and urine are grossly normal, but the spleen is greatly enlarged as is the gall bladder which usually contains a thick, sometimes even granular-like, bile. Moreover, the petechiation of organs found in many septicemias is absent. In leptospirosis, on the other hand, we find hemoglobinuria in addition to the splenomagaly, icterus, and anemia. The kidneys are petechiated, the liver is sometimes a gingerbread consistency, while the gall bladder and bile are relatively normal. In bacillary hemoglobinuria caused by Clostridium hemolyticum, a pathognomonic liver infarct is reported to be present. Generally speaking, the acute contagious anemias all present hemoglobinuria except anaplasmosis; and, in our practice anyway, only anaplasmosis manifests constipation.

Now, after having briefly reviewed the pertinent differential symptomatology, we must visualize a herd outbreak of anaplasmosis in order to appreciate its vicious destructiveness. The picture I present has been repeated many times in our practice, and it is a syndrome we dread to face.

Several years ago, a man purchased a ranch in our area and moved his entire herd of Hereford cows down from northern California. About 90 days after he arrived, he phoned to say that 3 cows had mysteriously died in the past week and that he had just discovered one who was very ill. On arriving at the ranch, I was asked to go on to the pasture as the riders were unable to bring the animal in. I went out to find a large eight-year-old Hereford cow holding two riders at bay. She was standing with head held high, pawing the ground, and turning from time to time to face one rider, then the other. She appeared irrational and definitely "on the fight." Her whole body was trembling and rigid, and her respiration was deep and labored. In her nervousness, she was constantly dribbling small amounts of normal looking urine and defecated some hard, mucus covered pellets. Her udder was icteric and numerous ticks could be seen on her body.

Suddenly she broke away and ran with a stilted, staggering gait. I told the riders not to follow. She ran about 60 yards, then stopped to turn and face us. In about a minute, she lay down. The riders approached her, and she attempted to regain her feet but was unable to do so. She was roped, a quick hemoglobin reading was taken, we glanced quickly at her icteric vulvar mucus membrane, and she was treated while struggling continously. Within three minutes she was released and we left her. She continued struggling, finally staggered to her feet, lurched about 30 yards, when her forelegs collapsed beneath her and she fell. When we approached, she was in a tetanic convulsion with her eyes rolled back and her mouth open struggling for air. Suddenly she relaxed and was dead.

I informed the owner he was in trouble, and he brought in the entire herd and sprayed for ticks.

Two days later, another case was found. She was treated and died. A day later, another case developed but we were prepared with whole citrated blood. She died before she had received 300 cc. The next day, another case was found and she received one gallon of whole blood. She never regained her feet. Four days later, another case died during the transfusion. The next day, another case collapsed and died before she could be roped. A week went by before we had another case. She was treated with drugs and died that night. Meanwhile three others had simply been found dead.

The owner became quite discouraged and wondered rather violently why, in this atomic age, the veterinary profession couldn't do something about this disease. This was about my second year in practice, and I wondered too. Anyway, in the next 90 days, this man lost 30 more cows, some which were treated and most of which were not. He didn't feel I was doing them much good, and I agreed.

Another ranch nearby has probably lost 250 shipped-in cows in the past 10 years. A third ranch in our hottest anaplasmosis belt often lost between 50 or 60 shipped-in cattle per season. We have used about every treatment described in the literature on these cases. On this third ranch, about five years ago, acute leptospirosis struck in the younger animals at the same time were battling anaplasmosis. There was no vaccine yet and the leptospirosis agglutination test wasn't too well developed. I found, though, that centrifuging a blood sample gave me a hemolyzed serum in leptospirosis and a non-hemolyzed serum in anaplasmosis. This test may not bear critical analysis but, in the yearlings who might have either disease, in the acute form; it at least gave us a clue for more accurate therapy. Another difficult chore was to treat all these animals where they were found. A good deal of our country is extremely rough, and I saw a good deal of it by jeep, on horseback, and on foot. For many reasons, then, I dispensed treatments to the owners whenever I could.

We have now described what I call the peracute form of the disease. We also have an acute form which is most common in our native cattle. Here we have

an animal who is not belligerent, and who may be driven slowly to a corral although she may rest occasionally along the way. Her blood hemoglobin may be below 4 grams per 100 ml serum, her stools are mucus covered pellets, and she can be exerted enough to kill her. But she won't usually try to kill herself. Her prognosis is quite good with modern treatments and even whole blood if it is available. Nearly all of our ranches have from two to a dozen of these cases annually in native cattle, and they are apparently animals who escaped becoming carriers at an early age.

Then there is a mild form. About three years ago, I examined a weaner calf for a client but could find very little wrong. The stool was normal, as was the blood picture, but there was a slight febrile reaction. A blood slide revealed some anaplasma bodies. No treatment was given and the calf recovered. Later, the owner informed that nearly the entire group of fifty calves revealed some weakness and depression at some time during the next 90 days. Apparently the terrific hemetopoietic powers of the young permitted them to maintain a status quo with the disease. I am informed, however, that these animals do die if violently exerted as during branding, castrating, and vaccinating.

This then is the story of clinical anaplasmosis in our practice. It is a disease we can treat successfully only in those animals who can or will conserve their depleted physical reserves until either the organism is inhibited or the results of a stimulated hematopoietic system are manifested. It is a disease in which therapy, no matter how controlled and ideal, is often wanting. It is a disease which, in our area anyway, requires some system of prophylaxis to reduce its heavy annual losses. This sorely needed prophylaxis, in endemic areas, is being accomplished now only by natural means and the surgical errors of man.

# THE LABORATORY DIAGNOSIS OF ANAPLASMOSIS

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The laboratory diagnosis of anaplasmosis is achieved by a recognition of those features which characterize the disease. The methods used for laboratory confirmation of a suspected case include: (1) the demonstration of typical *Anaplasma* bodies in the erythrocytes by microscopic examination of stained blood smears, (2) the post-mortem examination of the animal suspected of having the disease, (3) the serological detection of antibody in the blood serum by the complement-fixation (c.f.) test, (4) the inoculation of blood into splenectomized calves in order to demonstrate transmission of the disease, (5) the challenge inoculation of the suspected animal with virulent *Anaplasma* blood to demonstrate resistance to the disease, and (6) the removal of the spleen from a suspected carrier animal in order to determine the recurrence of *Anaplasma* bodies in the peripheral blood.

All of these methods have an important place in the diagnosis of the disease, as no one procedure is 100% accurate. A complete clinical history of the suspected animal and a history of the herd in question are of considerable value in making the diagnosis.

*Blood Examinations*.--Blood smears to be examined for *Anaplasma* bodies should be prepared with care and with clean slides. Smears submitted for laboratory examination are frequently not of sufficient quality to enable a satisfactory study. In order that the individual red blood cells can be examined, it is important that a thin film be made. The desired objective is to have a single layer of cells which do not touch each other. The freshly made blood film should be dried rapidly. Citrated or oxalated blood samples may be collected, and, if promptly sent to the laboratory, a number of films can be made under ideal conditions, as well as a hematocrit determination or an erythrocyte count. The dried blood film is fixed in methyl alcohol for five minutes. Commercially prepared concentrated Giemsa stain is diluted the day of use. Fifty drops of the stock staining solution is added, drop by drop, with agitation, to 50.0 ml. of distilled water buffered to pH 6.5. The blood films are held in the diluted staining solution for one hour and examined microscopically with the oil immersion objective.

Typical *Anaplasma* bodies appear as dense, round, dark-blue stained masses, varying in size from 0.3 to 0.9 of a microm in diameter. The bodies of *Anaplasma marginale* are located predominantly near the outer margin of the erythrocytes. In the acute case of anaplasmosis the bodies are usually seen for a period of 10 days to three weeks in the peripheral erythrocytes. Occasionally

a secondary rise and decline of bodies is seen in experimental cases of the disease. The percentage of infected cells may be estimated by counting the number of erythrocytes containing bodies seen during the examination of several hundred red blood cells. The percentage of infected cells increases rapidly in the early stage of the disease. This number will approximately double during each 24-hour period. After a peak is reached, the percentage of infected cells declines steadily.

Although the typical acute case of anaplasmosis is characterized by the presence of the Anaplasma bodies, the lack of such structures does not necessarily rule out the disease. As the anemia progresses, the percentage of infected cells declines rapidly. Eventually the bodies are undetectable, usually before recovery from the anemia occurs. Experimental cases of the disease have been observed in which the typical bodies did not appear. Recently we have observed two such cases. Both cases were suspected of being infected because of their significant serological reactions with the c.f. test. Inoculation of their blood into splenectomized calves resulted in a typical cases of the disease, with bodies appearing in the usual manner.

The use of blood smears for the diagnosis of carrier cases of anaplasmosis is of little value. Occasionally animals in the carrier state show a small number of bodies in their blood. However, this occurs only sporadically and would not be observed unless frequent bleedings were made. The red blood cells of non-infected cattle are sometimes observed to contain structures in small numbers similar to Anaplasma bodies. It is very difficult, and many times impossible on one examination to distinguish between these structures and true Anaplasma.

A determination of the packed red blood cell volume on a percentage basis, the hematocrit reading, is a valuable adjunct to other blood studies in cases of anaplasmosis. This method may be employed together with a red blood cell count or used alone to indicate the degree of anemia in an animal. The hematocrit reading varies in normal animals, usually from 28 to 42%. During the severe anemia stage in acute anaplasmosis the reading is often reduced to as low as 6-10%. This test is only indicative of the degree of anemia and is not specific for any single disease. The hematocrit reading in anaplasmosis carrier animals may be within the normal range.

Autopsy Findings.--The degree of pathological changes observed on autopsy depends upon the severity and stage of the disease. If death occurs from acute anaplasmosis, the characteristic findings are those seen in cases of marked destruction of red blood cells. The carcass may be somewhat emaciated and icteric; the blood is watery in appearance and thin in consistency; and the parenchymentous organs usually show degenerative changes. The outstanding findings are a greatly enlarged spleen, with very dark and soft pulp, a liver which is usually discolored and swollen, and a greatly distended gall bladder filled with dark, viscous bile. Scattered hemorrhages are seen on the heart and in the heart muscle. Gross pathological lesions are not prominent in the carrier case of the disease. However, numerous carrier animals which pass ante-mortem inspection as being free from

disease are found on post-mortem inspection to have a slight-to-moderate generalized icterus and an enlarged spleen. We have confirmed the c.f. test that many such animals, which are seen frequently in slaughtering establishments, are actually carrier cases of anaplasmosis.

The Complement-Fixation Test. --The c.f. test is used for numerous diagnostic procedures. The test utilizes the principle, discovered by Bordet and Gengou, that complement, the lytic factor of normal serum, can be used to demonstrate the reaction occurring between an antigen and antibody. Anaplasmosis antigen consists of a fraction of the lysed erythrocytes collected from an animal in the acute stage of the disease. The serum to be tested is mixed with an appropriate quantity of antigen and complement. If the serum contains anaplasmosis antibody, the complement is "fixed." This reaction, however, cannot be observed. Thus, a second mixture of sheep red blood cells and rabbit serum containing lytic antibodies for the sheep cells is added. If the complement was fixed by the first mixture of anaplasmosis antigen and serum, the sheep cells do not lyse and the test is positive for the unknown test serum. If, however, the test serum did not contain anaplasmosis antibody, the complement would be available for the second mixture and lysis of the sheep cells would occur. This would constitute a negative test on the unknown serum.

It is important that good specimens of sera be obtained for the c.f. test. The samples should be clear, preserved to prevent decomposition, and free from hemolysis. One or two ounces of whole blood is collected and allowed to clot. The clot may be "wrung" with sterile wire to facilitate the serum yield. After standing overnight in a warm room, the tube is centrifuged to clarify the serum. The sample is then poured off into another vial, and one part of 5% aqueous phenol is added to nine parts of serum, so that a final concentration of 0.5% phenol is obtained. Such samples may be shipped or held without spoilage and will provide satisfactory specimens for the c.f. test.

The routine qualitative c.f. test is a two-tube procedure. Serum is used in each tube; to one tube antigen and complement are added; to the other tube, only complement is added. This second tube is used to make sure the serum does not have anticomplementary properties, and in this case hemolysis occurs and is read negative. The tube containing antigen is used for the diagnostic reading. No hemolysis is read as 4+ fixation and indicates a positive reaction. A reading of 3+, 2+, or 1+ indicates the corresponding decrease in the degree of fixation, and the serum is reported as having a suspicious reaction. If the serum contains no antibody, the hemolysis is complete and the reaction is reported as negative. In the case of those sera having a positive reaction in the test, an estimate of the relative antibody present is made by making higher dilutions of the serum and repeating the test. The serum titer is expressed as the highest serum dilution which shows a 4+ reaction.

The c.f. test has been developed primarily to provide a method for the diagnosis of the carrier state of anaplasmosis. It has been demonstrated to be 95-98% accurate. The procedure can be rapidly conducted on a large number of

samples at one time, thus providing a means for detecting infected animals which could not be recognized by other methods, except by the laborious expensive and time-consuming animal inoculation tests. The test has a high degree of specificity. Serological cross-reactions do not occur with brucellosis, vibriosis, or piroplasmosis positive sera. However, other types of Anaplasma, such as the Anaplasma centrale strain and the Anaplasma ovis strain, do result in positive reactions to antigen made from Anaplasma marginale. The antibody level in the blood sera of carrier cases of anaplasmosis is of a relatively low magnitude, particularly in cases which have been infected for a long period of time. Therefore, in carrying out the test, the sensitivity must be of such a degree that these animals, as well as more recent cases having higher antibody levels, are detected. It is recognized that some nonspecific reactions do occur. We have observed such reactions in animals showing evidence of anemia from other causes. These nonspecific reactions are generally of the suspicious type and do not usually persist, in contrast to the continuous serological reaction obtained in cases of the infection.

Table I illustrates the development of Anaplasma bodies, the packed cell volume, and complement-fixing reactions in an experimental case of bovine anaplasmosis, from the time the animal was inoculated until several months later after it had progressed into the carrier state. This particular case is shown because it demonstrates the laboratory findings on a very mild form of the disease. The animal was a nonsplenectomized adult cow, which showed no external clinical manifestations during the acute phase of the infection.

Animal Inoculation Tests.--For purposes of confirming a diagnosis by animal inoculation, a splenectomized calf weighing 300 to 500 pounds is used. The removal of the spleen exhances the susceptibility of the young, naturally more resistant animal. Calves are obviously easier to handle and more economical to maintain than are adult cattle. Blood studies, including stained smears for microscopic examination, serum for the c.f. test, and hematocrit values, are made three times weekly in the test calf prior to and following splenectomy. Some splenectomized calves show heavy infections of Hemobartonella, Eperythrozoon, and Theileria. These infections complicate the blood picture and interfere with Anaplasma development unless they are allowed to clear up before the calf is used for a test. After the operation an interval of 4 to 6 weeks is allowed before inoculating the calf with suspected material. The test calf should be confined to insect proof isolation facilties. Citrated or defibrinated blood from the suspected case of anaplasmosis is used for inoculation. The blood should be collected and handled aseptically. It may be injected either subcutaneously or intravenously; however, we believe the intravenous route is preferable. The blood should be inoculated as soon as possible after collection. Volumes of 100 ml. or more should be employed. Large doses of infected blood provide a shorter incubation period and, more important, contribute to make the test more conclusive in the event of negative results. The test calf is observed and blood studies are continued for a period of 60 days. Twice a day temperature records are kept. The febrile response in calves inoculated with Anaplasma-infected blood if quite variable in duration and severity. If the test calf does not develop features characteristic of anaplasmosis during the 60-day

Table 1--Laboratory Studies of Anaplasmosis Cow 4231
(Not Splenectomized)*

| Date | Anaplasma Bodies | Red Cell Volume % | c. f. Test | c. f. Serum Titer |
|---|---|---|---|---|
| 2-7-57 | Negative | 30.0 | - | |
| 2-8 | " | 29.0 | - | |
| 2-11 | " | 33.0 | - | |
| 2-13 | " | 35.0 | - | |
| 2-15 | " | 31.0 | - | |
| 2-18 | " | 32.0 | - | |
| 2-20 | " | 34.0 | 1+ | |
| 2-22 | " | 30.0 | 4+ | 1:80 |
| 2-25 | " | 30.0 | 4+ | 1:160 |
| 2-27 | " | 34.0 | 4+ | 1:160 |
| 3-1 | 1.0% | 31.0 | 4+ | 1:160 |
| 3-4 | 6.0% | 23.0 | 4+ | 1:320 |
| 3-6 | 10.0% | 22.0 | 4+ | 1:320 |
| 3-8 | 10.0% | 16.0 | 4+ | 1:320 |
| 3-11 | 2.0% | 18.0 | 4+ | 1:320 |
| 3-13 | 1.0% | 19.0 | 4+ | 1:320 |
| 3-15 | Negative | 18.0 | 4+ | 1:320 |
| 3-18 | " | 24.0 | 4+ | 1:320 |
| 3-20 | " | 25.0 | 4+ | 1:320 |
| 3-22 | " | 25.0 | 4+ | 1:320 |
| 3-25 | " | 28.5 | 4+ | 1:160 |
| 3-27 | " | 26.0 | 4+ | 1:160 |
| 3-29 | " | 26.0 | 4+ | 1:160 |
| 4-1 | " | 28.0 | 4+ | 1:160 |
| 4-3 | " | 33.0 | 4+ | 1:80 |
| 4-5 | " | 33.5 | 4+ | 1:80 |
| 4-8 | " | 32.5 | 4+ | 1:80 |
| 4-10 | " | 33.0 | 4+ | 1:80 |
| 4-17 | " | 33.0 | 4+ | 1:40 |
| 4-24 | " | 33.5 | 4+ | 1:40 |
| 5-1 | " | 35.0 | 4+ | 1:20 |
| 5-8 | " | 38.0 | 4+ | 1:20 |
| 5-15 | " | 38.0 | 4+ | 1:20 |

*Inoculated subcutaneously Feb. 7, 1957, with 20 cc. citrated carrier blood collected from animal No. 3373

observation period, it is essential to determine its susceptibility or resistance.

Challenge Inoculation to Determine Resistance or Susceptibility. --Challenge inoculations with highly virulent blood may be made into either suspected field cases or artificially infected test calves to provide information regarding their infectivity status. The challenge inoculum should be standardization to the size of the recipient animal. The pathogenicity of the agent is increased for challenge purposes by making one or more passages in splenectomized calves until a parasitization of 50 to 60% is attained. At that time the blood is collected into citrate solution. As considerable toxic material may be present in the plasma, this is removed by centrifuging the packed red blood cells, decanting the plasma, and washing the packed red cells with physiological saline, and centrifuging again. The volume of packed cells is measured and suspended in an equal volume of physiological saline. Challenges are made using 0.5 ml. of packed red cells per pound of body weight for each test animal. The inoculation is given intravenously. Such a challenge results in severe manifestations of the disease in susceptible animals and affords a comparison of the degree in susceptibility in different animals. Carrier cases of anaplasmosis inoculated with virulent material show only a slight effect and very few Anaplasma bodies. The challenge dose described will cause death in a majority of susceptible animals. The chief weakness of the challenge method for determining the infectivity status of an animal is that additional subinoculation tests can no longer be made on that test animal after it has been challenged.

Tables II and III show the results of a challenge inoculation into two test calves which had been previously injected with material suspected of containing *A. marginale*. Neither test animal had shown any indication of being infected with anaplasmosis, and passage of their blood into other test calves proved negative. Other test animals challenged with the same material died between the 8th and 14th days.

Splenectomy of Suspected Carriers. --Another method which is used to determine whether an animal is a carrier of anaplasmosis is to remove the spleen and to observe the animal for indications of the disease. The effect of the splenectomy is to diminish the state of premunition of a carrier so that subsequent to the operation *Anaplasma* bodies will reappear in the peripheral red blood cells. This procedure has been used to confirm the infected status of suspected animals, especially when the subinoculation test gave negative results. The procedure is also of benefit in assuring the noninfected status of test animals which are being prepared for use in diagnostic or experimental studies in anaplasmosis work. If the test animals were not splenectomized before inoculation, this procedure offers an advantage over the previously described challenge method, because additional subinoculation tests could be made without having the known introduction of infection from another source.

We are constantly on the alert for more simple, rapid, accurate, inexpensive, and practical methods of diagnosing anaplasmosis. Some of the subjects on this program presented by other authors suggest such tests may be developed in the future. We attempt to use all the known diagnositc methods in our experimental studies, and even though they are expensive and time consuming, these methods have unlimited value in proving the weakness, limitations, and worth of other procedures.

Table II.--Challenge Results on Test Animal Demonstrating Susceptibility

Splenectomized Calf No. 4195 Challenged on March 21, 1957*

| Date | Anaplasma Bodies | Red Cell Volume % | c. f. Test | c. f. Serum Test |
|---|---|---|---|---|
| 3-22-57 | 3.0% | 31.0 | - | |
| 3-25 | 6.0% | 26.0 | - | |
| 3-27 | 31.0% | 24.0 | - | |
| 3-29 | 52.0% | 15.0 | 4+ | 1:40 |
| 4-1 | 21.6% | 9.5 | 4+ | 1:80 |
| 4-3 | 5.0% | 12.0 | 4+ | 1:80 |
| 4-5 | 0.5% | 20.0 | 4+ | 1:80 |
| 4-8 | Negative | 21.0 | 4+ | 1:80 |
| 4-10 | Negative | 23.0 | 4+ | 1:80 |
| 4-12 | Negative | 23.5 | 4+ | 1:80 |
| 4-15 | Negative | 27.0 | 4+ | 1:80 |
| 4-17 | Negative | 29.0 | 4+ | 1:80 |
| 4-19 | Negative | 30.0 | 4+ | 1:80 |
| 4-22 | Negative | 30.3 | 4+ | 1:40 |
| 4-24 | Negative | 33.0 | 4+ | 1:40 |

*Theileria mutans had been frequently observed during the 60-day period prior to challenge and occasionally thereafter.

Table III.--Challenge Results on Test Animal Demonstrating Resistance

Splenectomized Calf No. 4113 Challenged on March 21, 1957*

| Date | Anaplasma Bodies | Red Cell Volume % | c. f. Test | c. f. Serum Test |
|---|---|---|---|---|
| 3-22-57 | 0.5% | 27.5 | - | |
| 3-25 | 0.5% | 23.5 | - | |
| 3-27 | Negative | 24.5 | 4+ | 1:20 |
| 3-29 | Negative | 23.5 | 4+ | 1:20 |
| 4-1 | Negative | 23.0 | 4+ | 1:20 |
| 4-3 | Negative | 24.0 | 4+ | 1:20 |
| 4-5 | Negative | 22.0 | 4+ | 1:20 |
| 4-8 | Negative | 24.0 | 4+ | 1:20 |
| 4-10 | Negative | 24.5 | 4+ | 1:20 |
| 4-12 | Negative | 25.0 | 4+ | 1:20 |
| 4-15 | Negative | 26.0 | 4+ | 1:20 |
| 4-17 | Negative | 27.0 | 4+ | 1:10 |
| 4-19 | Negative | 25.7 | 4+ | 1:10 |
| 4-22 | Negative | 27.0 | 4+ | 1:10 |
| 4-24 | Negative | 28.0 | 4+ | 1:10 |

*Theileria mutans had been observed frequently during the 60-day period prior to challenge and occasionally thereafter.

# FIELD TRIALS WITH THE COMPLEMENT FIXATION TEST

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Unfortunately the application of the complement-fixation test for anaplasmosis in field trials to study the control and eradication of the disease has not been as widespread as we would desire. However, it has been used in some areas and has demonstrated that by testing the herds and removing the infected carrier animals for slaughter or by segregation, the spread of anaplasmosis can be controlled. It is certainly desired that such field trial studies be made in areas where there is a heavy incidence of ticks that are known to be vectors of the disease.

Some studies of this nature have been made in the State of Montana and will be reported by Dr. Tunnicliff.

Field trials with the complement-fixation test have been made in Virginia on a limited number of herds and these will be reported by Dr. Roby. The testing that has been accomplished there has led to the adoption of a voluntary anaplasmosis eradication program. The agreement between the owner and the Virginia Division of Animal and Dairy Industries for the eradication of the disease, outlining five requirements, is as follows:

1. Identification of all animals classified as reactors with segregation from the remainder of the herd.
2. The sale of those animals reacting to the test is limited to movement for slaughter.
3. The reactors shall move from the premise on a written permit obtained from the office of the state veterinarian.
4. The owner agrees to having his herd retested as required by the state veterinarian until the herd is declared free of anaplasmosis.
5. Herd additions must be tested and found negative prior to entry into the herd.

I am reporting this for Virginia as it is the only state with a formal anaplasmosis eradication program being offered.

The State of Washington has conducted some field trial studies in conjunction with their control and eradication efforts. When anaplasmosis is diagnosed, a state quarantine is placed on the herd allowing movements from the herd for slaughter only. Some owners have elected to feed out the entire herd for slaughter while others have followed a program of segregation and subsequent slaughter of reactor animals with herd retest. One such herd in the east central portion of the state was tested in July 1956 with the results of 136 negative animals and 28 reactors and 2

suspects. The reactor animals were segregated and later removed for slaughter. The herd was retested in November 1956 with 4 reactors and 1 suspect disclosed in 129 head. A spray program for vector control had been practiced during the summer months. The herd was again tested in April of 1957 with 5 reactors and 2 suspects disclosed. A group of 20 animals which had not been previously tested were included in this test and 3 of the 5 reactors were from this group.

Contact herds in the same general vicinity were tested. Of 7 herds tested only 1 disclosed anaplasmosis. Eight (8) reactors and 6 suspects and 67 negative animals were found on the first test in November 1956. After removal for slaughter of reactors and suspects, one reactor and one suspect were disclosed on the April 1957 test. Vector control programs will be continued in them this summer and further testing is planned.

Some complement-fixation testing has been accomplished on suspicious herds in Ohio since 1955. Four (4) herds have disclosed reactors. No formal program has been adopted there but the owners have been stimulated to dispose of the entire group for slaughter. The 4 infected groups found have been connected with importations from anaplasmosis areas.

Field trial studies for the application of the complement-fixation test have been inaugurated in the State of Tennessee in cooperation with the University of Tennessee and the state veterinarian's office. This work is being conducted in cooperation with the Animal Disease and Parasite Research Division and the Animal Disease Eradication Division. A number of herds are being tested, allowing the owner to decide whether he would like to operate his herd under one of three plans: (1) leaving the reactor and suspect animals in the herd to intermingle with the susceptible animals, (2) after test of the herd, the animals reacting from the test are segregated from the negative animals, and (3) the reactor animals will be removed from the herd and disposed of for immediate slaughter.

Reactor animals are being identified by tatoo and cannot be sold except under permit from the state veterinarian and only for immediate slaughter. It is hoped that sufficient herds with anaplasmosis in them can be disclosed and that approximately an equal number of herds and cattle can be operated under the three plans. So far, only 8 herds comprising of 391 animals have been signed up and tested under this field trial study. Two (2) of the 8 herds were negative on first test and only 2 of the reacting herds have been retested. One herd comprised of 56 head on original test disclosed 17 reactors and 7 suspects. On retest approximately one year later of the 29 head tested, there was 1 reactor and 1 suspect.

Another herd of 50 head had one reactor and one suspect. On retest of the 71 animals in the group at that time all were negative.

Eight (8) herds under the supervision of the University of Tennessee have been tested. Five (5) have disclosed positive animals and 3 have not. The infected herds have contained 1, 367 animals on the first test with 69 classed as reactors. There were 297 animals in the negative herds. Only 1 herd has been retested.

The first complete herd test was performed in February of 1956 when 574 animals were tested with 58 being classed as reactors and 7 as suspects. Thirteen (13) registered animals from the reactor group were retained and established as a segregated reactor herd. The remainder of the reactors and suspects were sold for slaughter.

The only vector control practiced was DDT and oil back-rubbers.

In December of 1956, the entire herd was retested, with 14 classed as reactors out of 602 head. One (1) new reactor was disclosed, as 13 of these were the retained reactors found on the first test.

Additional field trial studies using the complement-fixation test to disclose carrier animals are desired.

# FIELD TRIALS WITH THE COMPLEMENT FIXATION TEST

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The ideal method of attacking arthropod-borne diseases is eradication of the insect and arachnid vectors and/or elimination of the carrier focus from which the vectors receive the infection. Obviously it is the responsibility of research personnel to develop the necessary techniques for attaining these objectives.

It is widely recognized that no practical, effective control measures are known for the vectors of anaplasmosis. This is especially true in tropical and sub-tropical regions, and in those temperate regions where the various species of 3-host tick and blood-sucking insect vectors are active throughout the year.

Clinical anaplasmosis may and does occur during any month of the year in Florida herds, where all reasonable precautions are taken to prevent mechanical transmission through soiled instruments. Year-'round activity of anaplasmosis biological vectors and mechanical transmitters over the widespread acres mentioned offer formidable obstacles to effective control of the disease by eradicating vectors. Attention therefore must be focused on detection of carrier animals by means of available tests, either those we now have or those that can be developed. Such carrier animals, when identified, can be handled in a manner considered practical and applicable under environmental circumstances.

The complement fixation test for detecting anaplasmosis carrier cattle has been under limited field observation in Florida for approximately one year. The Florida station's serologist spent several weeks at the Agricultural Research Service's Anaplasmosis Research Laboratories in Washington where a knowledge was gained of the techniques developed and used by the scientists there. We are indebted to the Agricultural Research Service for this and other assistance in anaplasmosis investigations in our state.

On the basis of limited observations in Florida, it seems reasonable to believe that cattle in infected herds, reacting negative to the complement fixation test, when removed from the infected herd and placed on clean improved pastures where they are protected somewhat from exposure to infected vectors, can be maintained relatively free of anaplasmosis. This statement is based upon the general recognition that arthropod vectors do not travel great distances, that transmission is minimized under these conditions, and that many non-infected herds exist in Florida which are more or less in close proximity to or surrounded by infected herds.

The complement fixation test used in conjunction with fluorescent antibody technique offers addition possibilities which will prove of inestimable value in differentiation of infected from non-infected herds and in the final assault upon the anaplasmosis problem.

# FIELD TRIALS WITH THE COMPLEMENT FIXATION TEST

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The only herd that we have had an opportunity to apply control methods was our North Montana Branch Experiment Station, near Havre in north central Montana. Anaplasmosis was diagnosed in this beef herd in 1953. Since the objective of this experimental herd is production data and not disease study, it was not difficult to set up a program directed toward elimination of the disease.

The complement fixation test was used. All suspects and reactors (our 1-10 dilution) were either eliminated by slaughter or teated with an antibiotic.

A brief description of the situation should assist in an understanding of the problem. The herd is maintained in isolation at the Station during the winter, spring and fall. For the 3 to 4 months summer grazing period, the herd is taken south of the station into the Bear Paw mountains to a national forest allotment. This allotment is fenced with barbed wire and our cattle are contained therein, but separated on three sides from cattle owned by the Chippewa Cree tribe of Indians. These Indian cattle have a very high percentage of complement fixation anaplasmosis reactors. Obviously, at times there is close contact by the two groups of cattle and the presence of a wire fence does not provide a very formidable barrier for vectors ticks or flies. In spite of this flimsy barrier, transmission certainly hasn't been too rapid.

The first complement fixation test was made on this herd of cattle in the fall of 1953, at which time 333 cattle were tested, 315 being negative, 12 reacting and 6 were suspects. All suspects and reactors were eliminated or treated with antibiotics with the exception of bull 754. This animal gave a positive reaction on the first test September 1953, but was negative in October. Our interpretation was based upon the October test, so he was classed as a non-reactor and kept in the herd. Incidentally, he again gave a negative reaction in February of 1954.

When the herd was tested in December 1954, the entire herd consisting of 331 animals was negative with the exception of bull 754 that gave an incomplete complement fixation. Since he was a valuable breeding animal, it was decided to treat him with an antibiotic. This was done in January 1955. However, he did not lose the positive titre, was considered an infected carrier and sold in March 1955. This gave us an entire herd of non-reactors for the 1955 season. It must be remembered that he had been in contact with some cows during the breeding season the summer of 1954.

The herd test conducted in December 1955 gave 425 animal tests. There were 421 negatives, no reactors and 4 suspects. These four suspiciously reacting animals were slaughtered. Again the 1956 breeding season started with a non-reacting herd.

The 1956 herd test was conducted in December of that year. A total of 403 animals were tested, of which 401 were non-reactors and 2 were suspects. They were slaughtered. Again the 1957 season began with a non-reacting herd.

So far as clinical manifestations are concerned, none has been observed since the original outbreak in 1953.

# FIELD TRIALS WITH THE COMPLEMENT FIXATION TEST

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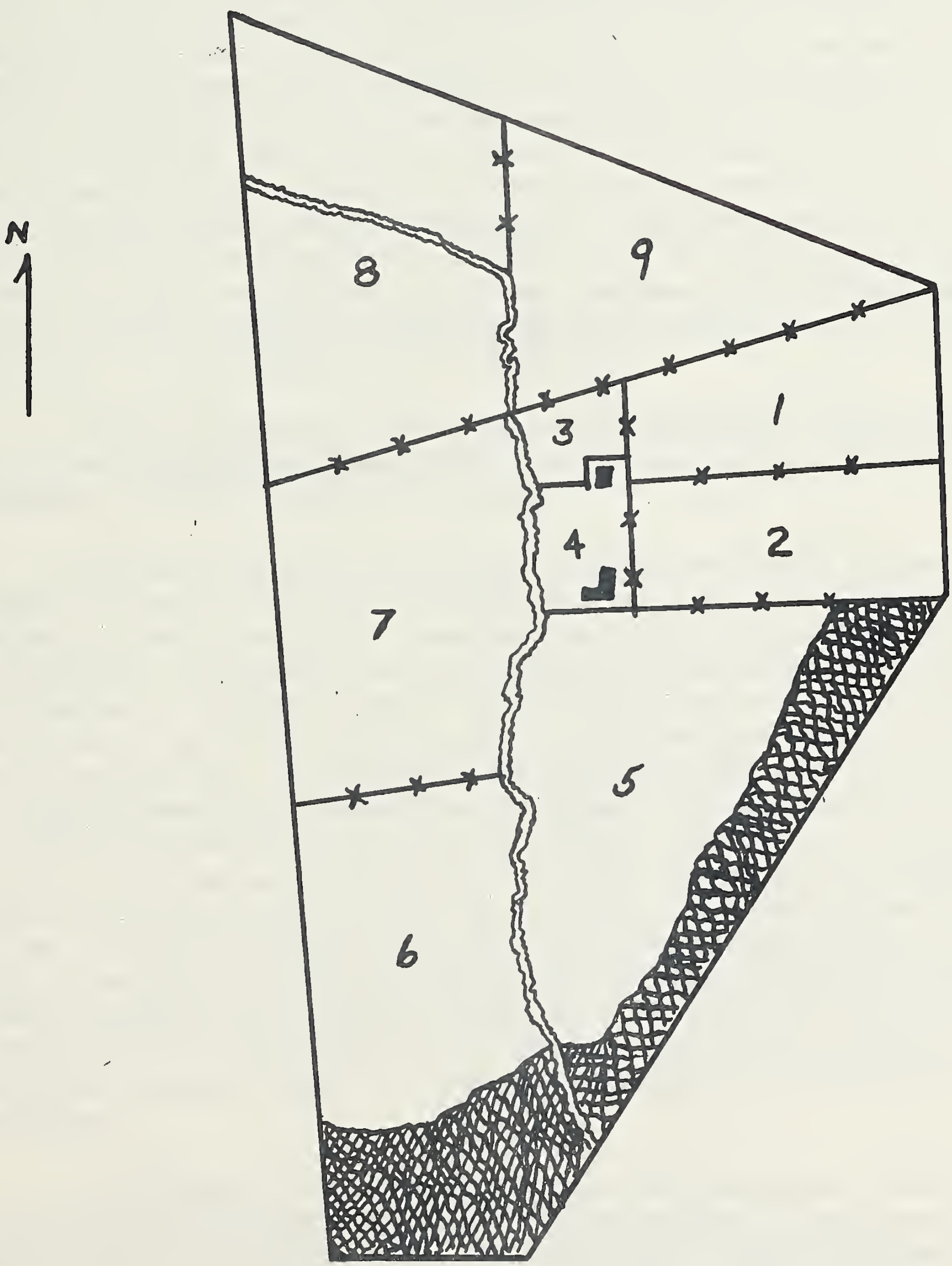
This presentation covers the studies which are being followed in three separate herds of cattle, two in Virginia and one in Texas.

Anaplasmosis has been eradicated in a beef herd in northern Virginia over a 4-year period by the application of the complement fixation (c.f.) test and a program of reactor segregation and disposal. This herd consisted of polled Herefords, home-raised, and was assembled during the years 1945-1950. In the spring of 1950 anaplasmosis was diagnosed in a steer 2 or 3 weeks after dehorning. The c.f. test at that time revealed 13 reactors and 5 suspects out of a total of 165 head. Two of the reactors were sold immediately, and the remaining 11 reactors and 5 suspects were segregated to area 6 (see farm layout map). A course of therapy was administered with an antimalarial chemical compound that had been reported to have some therapeutic value in anaplasmosis. Three months following the treatment, a retest of the reactors and suspects indicated little change in their serological status. After a retest of the entire herd in the fall, the reactors and suspects were sold for slaughter. The majority of these were adult cows. A c.f. negative bull that was turned out with the 16 reactors and suspects in area 6 in the spring was still negative on the fall retest. During the next 2 years, 1951-1952, no testing was done, as the owner thought the disease was eradicated.

In the fall of 1952, a cow developed anaplasmosis. The entire herd was tested in the spring of 1953, and 47 reactors and 14 suspects were found. It was decided by the owner to attempt a definite program designed to eventually eliminate the disease. Following the herd test in 1953, the reactor cows were pastured in area 8 and the nonreactor animals were pastured in areas 5, 6 and 7. The reactors and suspects were closely culled, and 26 were sold for slaughter. The nonreactor heifers were kept in area 1. All groups of cattle were sprayed with insecticides regularly, usually every 30 days, during the insect season. In the fall of 1953 about 35 of the reactors and suspects remained after culling, and these were wintered with the nonreactor cattle in a common feeding barn in area 4.

Annual retests have been made each spring on the animals which had

Virginia Farm Layout With Areas Where Anaplasmosis Negative and Reactor Cattle Were Kept Over A Three-Year Period



negative c.f. reactions the previous year. Table I indicates the results of these tests through 1957. After each herd test, new reactors were placed in the anaplasmosis herd and segregated during the insect season as before. The heifer calves from the reactor cows were placed with those from the nonreactors. By the spring of 1956 all previous c.f. reactor animals had been eliminated from the herd, and a herd test that year indicated no new reactors or suspects. The 1957 test also has been negative on the entire herd. Thus, it is apparent that segregation of infected cattle from non-infected cattle made this program successful. It was not possible to evalutate the role that regular spraying with insecticides had on disease control. The pooling of the reactor with nonreactor cattle during the winter is certainly not to be recommended, but was the only practical procedure for this particular farm. Moreover, the offspring of reactor cows ideally should have been tested upon weaning and found negative on two tests not less than 60 days apart before being placed with the clean group. Anaplasmosis has been reported by local practicing veterinarians for many years as a sporadic disease in this county. In fact, an outbreak of severe anaplasmosis occurred last summer (1956) in another beef herd approximately one mile from the location of the farm where the disease has been eradicated.

We are continuing to study both of these herds. The second herd is also under a segregation program in cooperation with the Bureau of Animal Industries, Commonwealth of Virginia.

Another beef herd in Virginia has been tested once annually for the last 3 years. This is a large herd of commercial Herefords, located in the south-western corner of the State. Anaplasmosis has not been previously recognized in the herd. The presence of the disease was confirmed by splenectomized calf inoculations in the fall of 1955. Table II indicates the results obtained on the three tests. All of the reactors and suspects were removed from the herd following the second test. It is planned that annual testing will be continued and that an anaplasmosis-free herd will be developed. During the last test, spring 1957, a 12-year-old bull was found to be a reactor. This animal's serum showed a titer of 1:160, indicating the acute or subacute stage of the disease. Citrated blood from the animal showed 2.0% infected cells and a hematocrit reading of 18.5%. Thus the evidence suggests that the bull became infected with anaplasmosis early in the spring of 1957 and raises the question as to the vectors responsible. Also, it points out the possible weakness in conducting only one annual test in an infected herd. Animals which may be in the incubation period of the disease may be missed. Retests made at 60-90-day intervals in an infected herd would be preferred.

We realize the need for eradication and segregation trials in areas where ticks are believed to be a major problem as vectors. While we do not know whether ticks are vectors of the disease in these Virginia herds, the anaplasmosis problem appears to be more severe in certain other areas of the South and West, where tick infestations are heavier.

Table I.--Results of Anaplasmosis Segregation Program in Small Beef Herd In Virginia Over a Four-Year Period

| Date | No. Tested | c. f. Results Negative | Suspicious | Positive | Disposition of Reactors and Suspects |
|---|---|---|---|---|---|
| 1953 April | 164 | 103 | 14 | 47 | 26 of the reactors and suspects sold |
| 1954 April | 95 | 90 | 0 | 5 | 35 reactors maintained |
| 1955 April | 109 | 107 | 2 | 0 | All reactors sold except 7 pregnant cows |
| 1956 April | 178* | 178 | 0 | 0 | Remaining 7 reactor cows sold |
| 1957 May | 136 | 136 | 0 | 0 | |

*Includes calves.

Table II.--Results of Three Annual Anaplasmosis Tests In a Large Beef Herd in Virginia Over a Two-Year Period

| Date | No. Tested | c. f. Results Negative | Suspicious | Positive | Anticomplementary |
|---|---|---|---|---|---|
| 1955 | 3178 | 3103 | 40(1) | 29(2) | 6(3) |
| 1956 | 2710 | 2681 | 7(4) | 20(5) | 2(3) |
| 1957 | 2654 | 2636 | 8 | 10(6) | 0 |

(1) 14 sold, 21 negative on retest, 5 positive on retest.

(2) 16 sold, 1 suspicious on retest, 10 positive on retest and 2 died.

(3) Negative on retest.

(4) 4 Spring calves negative on retest and calf subinoculation.

(5) 10 positive remaining from previous year's test.

(6) 1 Spring calf from reactor cow included.

The U. S. Department of Agriculture Entomology Research Station at Kerrville, Texas, maintains a herd of breeding cattle for production purposes. The incidence of anaplasmosis has been high in this herd, as judged by c.f. reactors. Clinical cases are also frequently seen. Plans are in progress to attempt the development of an anaplasmosis-free herd at this station. A pasture area will be divided so that the c.f. negative offspring of reactor cows can be removed to a separate pasture on weaning. At the present time the pasture area is being studied to determine the status regarding anaplasmosis of the native tick populations. Ticks are being collected off of the land and also off of the cattle and will be inoculated at Beltsville into splenectomized calves. The cattle presently on the pasture are also being tested serologically. It is expected that a program of at least 5 years will be necessary to determine if the transmission of the disease can be controlled under these conditions.

# ERADICATION OF ANAPLASMOSIS IN HAWAII

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Anaplasmosis was first diagnosed in Hawaii in May 1954 at a dairy in the Pearl City area, located about 10 miles northwest of Honolulu. The herd was known as the Brazil herd and totaled 130 head. A four year old Holstein cow imported from California the previous year, was found sick with clinical symptoms of anaplasmosis. Blood films revealed anaplasma-like bodies. Slides are forwarded to the Agricultural Research Service, Washington, D. C. for confirmation. The following day the animal was moribund. A blood sample was inoculated into a calf which had been splenectomized that morning. The cow was then euthanized, autopsied, and the cadaver incinerated. An inspection of the herd revealed no other sick animals, but a heavy louse infestation was noted.

This herd and three adjacent herds were placed under area quarantine. Insects were controlled by fogging and direct spray methods.

The experimental calf which was inoculated with blood from the sick cow showed anaplasma bodies in 17 days and died 5 days later. On the day of death, it had a total red cell count of 1,600,000/$mm^3$ and 5.0 gm/100 cc. hemoglobin. Autopsy revealed icterus, anemia, foam in trachea, sanguineous fluid in pericardial sac, mottled orange liver, thick floculent bile. A diagnosis of anaplasmosis was made.

This diagnosis was later confirmed by ARS following examination of the blood films originally submitted from the cow. While awaiting this confirmation, a request had been made to ARS for assistance in the detection of carriers by use of the complement-fixation test. Several weeks elapsed before approval was received.

In the meantime, calf trials were commenced on the entire Brazil herd using composite samples from approximately 20 animals into each splenectomized calf. When word was received that the ARS would conduct the complement-fixation test, blood samples were again drawn from the entire herd and the serum portions submitted to Washington for testing. Test results coincided completely with calf trial results as shown in Table I in that each calf developed anaplasmosis and test positive reactors were found in each group of cows that composed the source of inoculum. Other calves were inoculated from the three adjacent herds; none of these calves developed anaplasmosis and no test positive reactors were found in these herds.

On June 29, the Governor of Hawaii appointed an advisory committee on anaplasmosis to consult with the territorial veterinarian on plans to control and

TABLE 1
ORIGINAL QUARANTINED AREA
CALF INOCULATION TRIALS

| Calf No. | Source of Inoculum | No. Reactors to C.F. Test | Inoc. Date | Anaplasma on Smear | Disp. |
|---|---|---|---|---|---|
| 141 | Brazil 1 (20 animals) | 6 | 6/25/54 | 7/14/54 | 7/19-died |
| 143 | Brazil 2 (20 " ) | 3 | " | 7/19/54 | 7/25- " |
| 144 | Brazil 3 (20 " ) | 4 | " | 7/19/54 | 7/23- " |
| 150 | Brazil 4 (20 " ) | 5 | " | 7/14/54 | 7/18- " |
| 142 | Brazil 5 (21 " ) | 4 | " | 7/19/54 | 7/26- " |
| 149 | Brazil 6 (28 " ) | 3 | 6/26/54 | 7/19/54 | 7/23- " |

TABLE 2
SUMMARY OF TESTING - MAINLAND IMPORTS

| Period | No. Imported Dairy | No. Imported Beef | No. Negative Dairy | No. Negative Beef | No. Positive Dairy | No. Positive Beef |
|---|---|---|---|---|---|---|
| 8/1/54 - 12/31/54 | 713 | 35 | 694 | 35 | 19 | 0 |
| 1/1/55 - 6/30/55 | 481 | 49 | 481 | 49 | 0 | 0 |
| 7/1/55 - 6/30/56 | 1,983 | 86 | 1,982 | 81 | 1 | 5 |
| 7/1/56 - 5/31/57 | 2,018 | 163 | 2,014 | 163 | 4 | 0 |
| TOTALS: | 5,195 | 333 | 5,171 | 328 | 24 | 5 |

eradicate the disease. The committee was composed of five representatives of the beef and dairy industries and served as a buffer between the territorial veterinarian and industry groups. Recommendations to the advisory committee from the various cattlemen's associations ran the gamut of emotions from a demand to kill all cattle in the quarantine area to a laissez-faire' attitude of "so what's anaplasmosis". Fortunately, the committee members were level-headed men who could not be stampeded. To quiet the fears of the extremists, however, the committee recommended that guards be posted on the quarantine area and that an interisland quarantine be imposed to prevent the movement of cattle from the island of Oahu to the other islands. These recommendations were put into effect July 1, 1954.

An agreement was reached with ADPR to continue the Brazil herd testing provided all reactors were immediately sent to slaughter and the herd submitted for retest at 60-day intervals until two negative tests had been obtained.

Inquiry revealed that a majority of the reactors found in the Brazil herd had been imported from a single herd in California in September of the previous year. This shipment had been split between the Brazil herd and another herd located approximately 12 miles from Honolulu in a southeasterly direction. Permission was requested and granted to test this second herd. This test revealed 15 reactors, 14 of which were among 18 animals purchased from the infected shipment. Further investigation revealed that at different times other dairymen had imported cattle from the same herd in California and from adjacent herds in the same area. Permission was therefore requested and approval received from the ADPR to submit samples from these other herds.

As the expanded-testing information accumulated, it was found that test positive reactors were present in a number of other herds and information as to origin of these reactors indicated that the majority were imported from the state of California. Therefore, it became evident that any control program would have to include the testing of imported cattle. At the AVMA convention in Seattle in August 1954, discussions with Dr. H. W. Schoening, head of ADPR, and the state veterinarians of Washington and California resulted in an agreement for ADPR to test serum samples submitted to Washington on cattle intended for importation into the Territory of Hawaii. Subsequently, the regulation governing importation of cattle into the Territory was amended to include this test requirement. Imported cattle were required to be test negative within 30 days of shipment and were placed in quarantine upon arrival for retesting. They were also put through a spray-dip machine and sprayed with 2 1/4% DDT and .25% chlordane insecticidal solution. Results of testing the imports in quarantine to date are given in Table 2.

At Dr. Schoening's suggestion, our veterinary pathologist was sent to Washington to study the complement-fixation test. Commencing March 14, 1955 all tests were run in the Hawaiian laboratory using antigen furnished by ADPR. Duplicate serum samples were submitted to Washington for comparative testing. This comparative testing was continued on all serum samples collected

until it was decided that test results in the Hawaiian laboratory were in sufficient agreement with the results in the ADPR laboratory to permit the acceptance of the negative results obtained in Hawaii. After that all sera showing reactions in any degree or that were anticomplementary were submitted to Washington together with an equal number of negative sera from the same herds for comparative testing. This procedure is still being followed. A summary of the results of comparative testing to May 31, 1957 is given in Table 3.

Although some blood samples collected at slaughter had been included in the early survey testing, it was not until after the Hawaiian laboratory was staffed and equipped to conduct the complement-fixation test that it was decided to collect blood samples from all cattle slaughtered in the Territory for testing as a continuing survey procedure. This procedure was relatively simple to put into effect because Hawaii has compulsory meat inspection. Personnel of the bureau of meat inspection of the division of animal industry collect the blood samples at slaughter and forward them to the laboratory in Honolulu. Results of this survey testing are given in Table 4.

The testing of the Brazil herd continued at approximately 60-day intervals until two negative tests were obtained. The herd test pattern is as shown in Table 5. At the time that the bleedings were made for the 4th retest, blood samples were also drawn, composited, and inoculated into three splenectomized calves. These calves were observed for 60 days with negative results, thus, in effect, confirming the complement-fixation test results. The quarantine on the Brazil herd was then lifted. The herd was subsequently moved to a new location. Two annual retests have revealed no reactors.

Transmission trials from reactors in herds outside the original quarantine zone indicated that the test occasionally produced false positive results; however, no evidence of false negative results was uncovered. The occurrence of occasional false positive results failed to disturb our confidence in the complement-fixation test as a reliable tool for the detection of carrier animals of anaplasmosis. It was therefore decided that if we accepted the reliability of the complement-fixation test for the detection of carriers of anaplasmosis and took into consideration the following facts and conditions, a test and slaughter eradication program was feasible in Hawaii:

1. Insularity. Because of its geographic position, Hawaii was able to thoroughly and carefully screen all animal imports.
2. Vectors. The absence of important vectors of anaplasmosis in Hawaii was a favorable factor.
3. Auction Sales. There were no auction sales organizations. Normal movement of cattle was direct from farm to market.
4. Separation of Counties. Each county in Hawaii was separated from the others by a large expanse of ocean which constituted a natural barrier to the uncontrolled movement of livestock between counties.
5. Clinical Anaplasmosis. The absence of clinical disease in the presence of known carriers was considered a favorable factor.

**TABLE 3**
SUMMARY OF COMPARATIVE TESTING
HAWAII - WASHINGTON LABORATORIES

| Period | TECHNIQUE DIFFERENCES HAWAII: 2 units comp. to titrate antigen | Total Vol. Ant./Test | Pos. Serum Titer higher | No. Samples Tested | No. Agree | No. Samples Disagree: 1 ≠ Diff. | 2 ≠ or more |
|---|---|---|---|---|---|---|---|
| 11/1/55-12/30/55 | | .3cc/.45cc | | 137 | 94 | 28 | 15 |
| 1/1/56-3/26/56 | | .3cc | | 117 | 73 | 17 | 27 |
| 3/27/56-4/10/56 | | 0.75cc | | 87 | 49 | 13 | 25 |
| 4/11/56-4/30/56 | | .45cc | | 98 | 73 | 9 | 16 (18.90%) |
| | | | | After technique and procedure adjusted | | | |
| 5/1/56-6/30/56 | 1.5 units | | | 315 | 208 | 49 | 58 |
| 7/1/56-9/30/56 | | | | 3,545 | 3,154 | 180 | 211 |
| 10/1/56-12/31/56 | | | | 105 | 71 | 25 | 9 |
| 1/1/57-4/30/57 | | | | 124 | 85 | 22 | 17 ( 7.21%) |
| TOTALS: | | | | 4,578 | 3,807 | 343 | 378 ( 8.25%) |

TABLE 4
SUMMARY OF SURVEY TESTING OF SAMPLES COLLECTED AT SLAUGHTER
PERIOD: 11/54 - 6/30/56, 7/1/56 - 5/31/57

| DAIRY CATTLE Island | Period | No. Herds | No. Tested | No. Neg. | No. Pos. | Other |
|---|---|---|---|---|---|---|
| Oahu | 54-56 | 54 | 2,310 | 2,291 | 12 | 5 susp., 2 a.c. |
| | | *B.Y. 1 | 8 | 7 | 1 | |
| | 56-57 | 6 | 3,252 | 3,235 | 9 | 7 susp., 1 a.c. |
| Hawaii | 54-56 | 5 | 138 | 138 | | |
| | | *B.Y. 1 | 1 | 1 | | |
| | 56-57 | 1 | 38 | 38 | | |
| | | *B.Y. 1 | 1 | 1 | | |
| Maui | 54-56 | 1 | 224 | 222 | 2 | |
| | 56-57 | 2 | 82 | 82 | | |
| Molokai | 54-56 | 0 | 0 | 0 | | |
| | 56-57 | 1 | 3 | 3 | | |
| Kauai | 54-56 | 1 | 69 | 69 | | |
| | 56-57 | 4 | 70 | 70 | | |
| Total: | | 75 | 6,186 | 6,148 | 23 | 12 susp., 3 a.c. |
| | | *B.Y. 3 | 10 | 9 | 1 | |
| **BEEF CATTLE** | | | | | | |
| Oahu | 54-56 | 13 | 3,785 | 3,765 | 9 | 9 susp., 2 a.c. |
| | | *B.Y. 222 | 816 | 812 | 2 | 1 susp., 1 a.c. |
| | 56-57 | 3 | 2,364 | 2,359 | 1 | 4 susp. |
| | | *B.Y. 489 | 1,013 | 1,011 | 1 | 1 susp. |
| Hawaii | 54-56 | 43 | 27,610 | 27,464 | 73 | 32 susp., 41 a.c. |
| | | *B.Y. 378 | 1,879 | 1,873 | 3 | 2 susp., 1 a.c. |
| | 56-57 | 5 | 19,537 | 19,515 | 9 | 12 susp., 1 a.c. |
| | | *B.Y. 325 | 1,583 | 1,580 | 0 | 3 susp. |
| Maui | 54-56 | 16 | 5,766 | 5,758 | 1 | 5 susp., 2 a.c. |
| | | *B.Y. 71 | 419 | 419 | | |
| | 56-57 | 2 | 5,637 | 5,633 | 3 | 1 susp. |
| | | *B.Y. 160 | 841 | 841 | | |
| Molokai | 54-56 | 7 | 1,331 | 1,328 | 1 | 1 susp., 1 a.c. |
| | | *B.Y. 29 | 148 | 148 | | |
| | 56-57 | 1 | 704 | 703 | 1 | |
| | | *B.Y. 2 | 13 | 13 | | |
| Kauai | 54-56 | 14 | 2,366 | 2,362 | 0 | 1 susp., 3 a.c. |
| | | *B.Y. 128 | 313 | 312 | 0 | 1 susp. |
| | 56-57 | 2 | 2,253 | 2,246 | 5 | 2 a.c. |
| | | *B.Y. 165 | 722 | 721 | 1 | |
| Total: | | 106 | 71,353 | 71,133 | 103 | 65 susp., 52 a.c. |
| | | *B.Y. 1,969 | 7,747 | 7,730 | 7 | 8 susp., 2 a.c. |

*B.Y. - Backyard

TABLE 5
BRAZIL DAIRY

| Test Date | Type Test | No. Tested | No. Negative | No. Reactors |
|---|---|---|---|---|
| 6/28/54 | Initial | 129 | 104 | 25 |
| 8/25/54 | Retest #1 | 100 | 98 | 2 |
| 10/22/54 | Retest #2 | 92 | 91 | 1 |
| 12/27/54 | Retest #3 (incl. test negative additions) | 120 | 120 | 0 |
| 3/14/55 | Retest #4 | 117 | 117 | 0 |
| 5/17/56 | Retest after 1 year | 172 | 172 | 0 |
| 6/1/57 | Retest after 2 yrs. | 153 | 152 | *(1 suspect) |

*Note: Pending ADPR confirmation. This animal was a test negative herd addition which gave a suspicious reaction on retest.

6. Rate of Spread. Test information indicated that the rate of spread from imported carriers to susceptible animals was very low.

When this information was presented to the cattlemen in the Territory, most agreed that an eradication program should be undertaken. A few argued that the cost would be too high and others claimed that by barring further imports of carriers that the carrier animals already in the Territory would gradually be eliminated by normal marketing attrition. The cost argument was countered with the highly probable fact that at no foreseeable future time could eradication be done for less. The proponents of the gradual attrition program were confronted with the argument that the existing rate of spread could balance or could overcome the rate of attrition and, in addition, that a substantial hazard would ever be present so long as carriers remained in the Territory because our entomological records show that 15 to 20 emigrant insects are being found in Hawaii annually, making it highly probable that a known or unknown vector of anaplasmosis could gain admittance at any time.* Thus, general agreement was reached upon the desirability and feasibility of eliminating the carrier animals by test and slaughter. Repeated requests were therefore submitted to the Agricultural Research Service for assistance in conducting a test and slaughter field trial in Hawaii. These requests culminated in an invitation to attend the anaplasmosis conference held in Chicago on February 28, 1955 where consultations with Drs. M. R. Clarkson, R. J. Anderson, and L. O. Mott resulted in a tentative agreement to conduct a cooperative eradication program.

The Hawaiian legislature authorized such a program and appropriated $90,000 for the biennial period starting July 1, 1955. The legislation fixed the amount of indemnity to be paid the owner of reactor cattle ordered to slaughter at 1/3 of the difference between the appraised value and slaughter salvage, not to exceed $100.00 per animal. Authority to control diseases of domestic animals is delegated by law to the Board of Commissioners of Agriculture and Forestry, subject to the approval of the governor. Public hearings are held after which the Board adopts the necessary regulations having full force and effect of law. Such a regulation to eradicate anaplasmosis was adopted by the Board on September 29, 1955 and approved by the governor on October 13, 1955. The effect of this regulation was to require the testing of all cattle which had been imported prior to January, 1955 and which had not been previously tested; require all dairy cattle to be tested for anaplasmosis and all other cattle in herds in which evidence of infection is found in the survey testing. It requires that reactors shall be slaughtered within 30 days and sets forth a method of appraisal and indemnification; requires that all herd additions have two successive negative tests at 60-day intervals unless they have originated

* On January 6, 1957, ticks were found on a horse which had entered the Territory from Montana three weeks previously. A tentative identification of the ticks as Dermacentor albipictus made in Hawaii was confirmed by specialists of ARS. An eradication program was carried out which 5 months later seems to have been successful. This demonstrates the validity of the declaration of continuous hazard that will exist so long as carriers of anaplasmosis remain in the Territory.

in an anaplasmosis free herd, and restricts the interisland movement of cattle to test negative animals. A memorandum of understanding between the United States ARS and the Hawaii Board of Commissioners of Agriculture and Forestry implemented the program from the federal standpoint and authorized payment of matching indemnity funds out of the federal treasury. The cooperative program became effective November 16, 1955.

On November 1, 1955 a private practitioner brought a blood sample into the laboratory from a sick cow which, on stained smear, revealed anaplasmosis bodies on the red cells and gave a positive reaction to the complement-fixation test. The animal was one of 507 head in a large dairy herd located about 25 miles north of Honolulu. The sick animal subsequently died, and the herdsmen, when questioned, recalled several similar cases having recently occurred in the herd, one of which recovered from the disease. This recovered animal, when tested, proved to be a reactor. Permission was received from the ADE Division to proceed with the official program in this herd so long as actual indemnification of reactors was not made prior to November 16, 1955, the effective date of the program. The entire herd of 506 animals was immediately tested and four reactors were found. The cattle were also found to be quite heavily infested with lice. It was believed that the lice were responsible for transmission of the disease within the herd and a program of louse eradication was inaugurated. On December 12 another clinical case was observed in this herd. This animal developed typical symptoms of the disease and was found positive to the complement-fixation test, and anaplasma bodies were demonstrated in stained blood smears. The animal survived the illness, was appraised, branded and ordered to slaughter. The herd was retested in January and only one reactor revealed; a second retest in March was negative except for one suspect which on retest proved to be negative. A third retest conducted in May resulted in two suspects which on further retest proved negative. The herd test pattern was as shown in Table 6. Further herd tests have not been made up to the closing date of this report, however, since the last retest, when the herd was composed of 415 animals, 113 have gone to slaughter and have been test negative at that time. 98 head have been added to the herd during the same period. Each of these animals were imports and test negative prior to importation, were test negative in quarantine on arrival in the Territory, and were again negative 60 days after arriving on the farm.

In January 1956 an ADE Division field veterinarian arrived in Honolulu assigned to the cooperative program. By June 30, despite several months of inclement weather, all dairies in the Territory had received at least one test and scheduled retests were kept current. Results of all testing to June 30, 1956 are given in Table 7, Part A. Table 7, Part B, gives the testing results from July 1, 1956 to May 31, 1957.

An analysis of the testing results to May 31, 1957 as applied to individual dairy herds is given in Table 8.

TABLE 6
HYGIENIC DAIRY

| Test Date | Type Test | No. Tested | No. Negative | No. Reactors |
|---|---|---|---|---|
| 11/10-17/55 | Initial | 506 | 502 | 4 |
| 1/19/56 | Retest #1 | 476 | 475 | 1 |
| 3/9-13/56 | Retest #2 | 444 | 444 | 0 |
| 5/29/56 | Retest #3 | 415 | 415 | 0 |

TABLE 7
RESULTS OF COMPLEMENT-FIXATION TESTS FOR ANAPLASMOSIS

PART A (Period: 6/1/54 - 6/30/56) | PART B (Period: 7/1/56 - 5/31/57)

| | Part A Dairy Herd | | Part A Beef Herd | | Part B Dairy Herd | | Part B Beef Herd | |
|---|---|---|---|---|---|---|---|---|
| Island | No. Tests | Reactors | No. Tests | Reactors | No. Tests | Reactors | No. Tests | Reactors |
| OAHU | 6,566 | 322 | 1,470 | 3 | 19,883 | 67 | 828 | 0 |
| HAWAII | 1,979 | 10 | 6,229 | 33 | 1,904 | 0 | 6,361 | 3 |
| MAUI | 2,465 | 2 | 1,118 | 1 | 318 | 0 | 2,991 | 1 |
| MOLOKAI | 105 | 0 | 84 | 1 | 49 | 0 | 251 | 0 |
| KAUAI | 1,642 | 3 | 289 | 0 | 681 | 0 | 221 | 0 |
| TOTALS: | 12,757 | 337 | 9,190 | 38 | 22,835 | 67 | 10,652 | 4 |

TABLE 8
DAIRY HERD TESTING

| Type Test | No. Herds | No. Cattle | No. Reactors |
|---|---|---|---|
| Initial | 93 | 14,750 | 127 |
| Retest 1 | 79 | 13,636 | 35 |
| Retest 2 | 36 | 8,451 | 31 |
| Retest 3 | 15 | 4,424 | 3 |
| Retest 4 | 5 | 2,172 | 3 |
| Retest 5 | 1 | 190 | 0 |

| DAIRY HERD STATUS | *No. Animals |
|---|---|
| 1 Herd clean (original infected herd - 2 retests annually after lifting of quarantine) | - 153 |
| 46 Herds with 2 clean tests - no reactors | - 4,822 |
| 27 Herds with 2 successive clean tests after removal of reactors | - 4,883 |
| 1 Herd with 1 clean test after removal of reactors | - 158 |
| 15 Herds with initial test - no reactors | - 827 |
| 2 Herds with reactors on last test | - 1,836 |
| 1 Herd dissolved | -- |
| 93 Herds | 12,679 |

(*Note: Number involved on last test)

The field work progressed through the initial herd testing phase without untoward incident. On first retest, a large herd which had been negative on initial test revealed reactors as shown in Table 9. No additions were made to the herd between the initial and first retest. The reactor animals were recorded as herd members on the initial test. All cattle adjacent to the herd were test negative. No source of infection could be uncovered.

A similar, although not parallel, situation was found in the Moanalua herd. The test pattern to date is shown in Table 10. This herd was first tested in January 1955 during the survey period prior to inauguration of the cooperative program, and, as indicated above, was negative except for one 3+ suspect that was voluntarily slaughtered on February 1st. On the first test under the cooperative program which was made 13 months later, three reactors were found. Investigation revealed that these reactors were in the herd at the time of the first test and that all later herd additions were test negative. The herd was also noted as having a fairly heavy louse infestation. Correspondence with Dr. Mott of ADPR in search of an explanation for the developments observed in the Kaneohe and Moanalua herds focused our attention on our test procedure. As will be explained later, the reactors were probably missed on the first test in the Hawaiian laboratory because of a fault in test technique.

On second retest of herds, a different kind of problem was presented. The test pattern of the Campos herd illustrates this problem (Table 11). The first test revealed 24 reactors and 1 suspect. Permission was received to include the suspect with the reactors. All 25 animals were sent to slaughter. On the first retest, only 1 reactor was found and we therefore expected the second retest to be negative. To our surprise, 12 reactors were uncovered. No untested additions had been made to the herd and there were no untested adjacent cattle; no clinical evidence of anaplasmosis had been observed. An examination of the herd revealed a heavy louse infestation. A louse control program was immediately instituted by spraying the cattle as well as barns, runways, chutes and corral fences. The results of subsequent testing indicate that the louse was the probable cause of the increase in reactors on second retest. After this experience, it was decided that all herds containing reactors and which were found to have a louse infestation would be de-loused at government expense. It was considered more rational to buy insecticides than to pay indemnities. We are convinced that this policy has considerably influenced the success of our program.

## PROBLEMS ENCOUNTERED IN TEST TECHNIQUE AND PROCEDURE

Work in progress at ADPR, together with an analysis of the Hawaiian testing data and calf inoculation trials accumulated to the end of 1954, suggested possible need for revision of test technique and interpretation. Subsequently, Dr. Mott instructed s to increase the amount of complement used in the test procedure from 1.5 to 2 units and to change the test interpretation on the 3+ reactions from positive to suspicious. No further changes have been made on these points.

TABLE 9
KANEOHE DAIRY

| Test Date | Type Test | No. Tested | No. Negative | No. Reactors |
|---|---|---|---|---|
| 12/1/55 | Initial | 248 | 248 | 0 |
| 2/29/56 | Retest #1 | 228 | 225 | 3 |
| 5/11/56 | Retest #2 | 217 | 217 | 0 |
| 8/15/56 | Retest #3 | 202 | 202 | 0 |
| 2/10/57 | Retest of Additions | 48 | 48 | 0 |

TABLE 10
MOANALUA FARMS

| Test Date | Type Test | No. Tested | No. Negative | No. Reactors |
|---|---|---|---|---|
| 1/14/55 | Survey | 602 | 601 | (1 susp.) |
| 3/12/56 | Initial | 805 | 802 | 3 |
| 5/28/56 | Retest #1 | 807 | 804 | 1 (2 susp.) |
| 8/9/56 | Retest #2 | 795 | 792 | 3 |
| 12/11/56 | Retest #3 | 818 | 816 | 2 |
| 4/26/57 | Retest #4 | 787 | 784 | 3 |

TABLE 11
CAMPOS RANCH

| Test Date | Type Test | No. Tested | No. Negative | No. Reactors |
|---|---|---|---|---|
| 12/12/55 | Initial | 819 | 794 | 25 |
| 2/29/56 | Retest #1 | 762 | 761 | 1 |
| 5/8/56 | Retest #2 | 772 | 760 | 12 |
| | Louse Control Program Instituted | | | |
| 8/31/56 | Retest #3 | 769 | 769 | 0 |
| 1/22/57 | Retest #4 | 843 | 843 | 0 |
| | Herd Additions (60-day retest) | 76 | 76 | 0 |
| Since 1/22/57 | Slaughter Samples | 62 | 62 | 0 |

When the Hawaiian laboratory started testing the test procedure did not require phenolization of serum samples as a part of the technique, however, it was suggested by ADPR that it would be a good idea to preserve the samples when forwarding them to Washington because of the length of time the serum would be in transit. Later, it was learned that the addition of .5 percent phenol was not only recommended for the preservative action, but that it was actually essential to the test procedure to obtain accurate reproduceable results on some sera. In reporting results of some studies of this phenomenon, Dr. Mott made the following statement: "It appears that not only is the phenol needed for the preservative to keep down bacterial contamination, but it is also needed to sensitize the weakly positive serum. We have noticed the sensitization effect of phenol on serum for other complement-fixation tests in years past but never with this degree of difference."

Some work undertaken in the Hawaiian laboratory to determine whether or not the time of phenolization after drawing of the blood samples had any effect on the test gave the results shown in Table 12.

A little later we found time to try variations in the amount of phenol on the same serum sample (Carrier #142) as shown in Table 13.

Later in 1955, we learned how important it is to standardise the test technique and components of the test when the work is being done in two separate laboratories. Our comparative testing results with the ADPR laboratory had not been entirely satisfactory. The Hawaiian laboratory had been using commercial lyophylized complement because of a shortage of guinea pigs, whereas the ADPR laboratory was using fresh-frozen guinea pig serum. In the course of comparing results obtained with the two types of complement, we also learned that for best results an interval of time should elapse between phenolization and testing. In the Hawaiian laboratory, due to force of circumstances, it had been usually necessary to test the serum immediately after phenolization. Table 14 illustrates these two points.

Even after these adjustments had been made in the test technique, results of comparative testing between the two laboratories were still unsatisfactory. Up until this time, the Hawaiian laboratory was using 1.5 units of antigen per test based upon titrations done in the Hawaiian laboratory and without reference to titrations made on the same antigen at Beltsville or to the total quantity of antigen being used per test at Beltsville. After considerable correspondence and double checking of the test procedure as carried out in the Hawaiian laboratory, it came to light that the antigen titrations being conducted in the Hawaiian laboratory were being made by using two units of complement instead of 1.5 units as was being used in Beltsville. This error came about when it was earlier decided to increase the complement in the test proper from 1.5 to 2 units. While working out this problem, it was also learned that the Hawaiian laboratory was using a positive control serum of somewhat higher titer than that being used in the Beltsville laboratory. The Hawaiian serum was obtained from a carrier animal which had been maintained for that purpose. At Dr. Mott's suggestion the serum from

TABLE 12

(Carrier #142 (Titer 1:5) was bled and serum left with the clot. Each day enough serum was taken to run one phenolized and one non-phenolized sample.)

| Age of Sample | Phenol | No Phenol | Age of Sample | Phenol | No Phenol |
|---|---|---|---|---|---|
| 1 day | 4+ | ± | 9 days | 3+ | + |
| 2 days | 4+ | ± | 12 " | 4+ | + |
| 6 " | 4+ | + | 13 " | 4+ | + |
| 7 " | + | ± | 14 " | 4+ | + |
| 8 " | 4+ | 3+ | 15 " | 4+ | 2+ |

TABLE 13

| Tube | Method of Treatment | 11/29 | 11/30 | 12/1 | 12/2 | 12/5 | 12/6 |
|---|---|---|---|---|---|---|---|
| A | No phenol | - | - | - | - | 4+ | ± |
| B | 1/2 normal amt. of phenol | - | 2+ | 2+ | 2+ | 3+ | + |
| C | Normal amount of phenol | - | 3+ | 3+ | 3+ | 4+ | 3+ |
| D | $1\frac{1}{2}$ normal amt. of phenol | ± | 3+ | 4+ | 4+ | 4+ | 3+ |
| E | Twice normal amt. of phenol | 3+ | 4+ | 4+ | 4+ | 4+ | 4+ |

TABLE 14
LYOPHYLIZED/FRESH GUINEA PIG SERUM
IMMEDIATE TESTING AFTER PHENOLIZATION/DELAYED TESTING

| Sample No. | Hawaii Immed. after Phenol./Lyoph. Comp. Date | Results | Wash./Fresh Frozen Guinea Pig Serum Date | Results | Haw'n Test - 1/6/56 Original Serum Sample Lyoph. | Fresh G.P.Serum |
|---|---|---|---|---|---|---|
| KU-8 | 11/18 | 3+ | 12/15 | 4+ | 4+ | 4+ |
| KU-19 | | 3+ | | 4+ | 4+ | 4+ |
| PUO-25 | 11/21 | 2+ | 11/23 | 4+ | 4+ | 4+ |
| HT-156 | 11/25 | 2+ | | 4+ | 3+ | 4+ |
| S-13 | 11/30 | 3+ | 12/5 | 4+ | 4+ | 4+ |
| S-69 | | 3+ | | 4+ | 4+ | 4+ |
| S-70 | | 2+ | | 4+ | 4+ | 4+ |
| C-211 | 12/7 | 2+ | 12/7 | 4+ | 4+ | 4+ |
| C270 | 12/8 | 2+ | | 4+ | 4+ | 4+ |
| C-369 | | 2+ | | 4+ | 4+ | 4+ |
| C-400 | | 2+ | | 4+ | 4+ | 4+ |
| C-402 | | 2+ | | 4+ | 4+ | 4+ |
| C-448 | 12/9 | 2+ | | 4+ | 3+ | 4+ |
| C-542 | | 2+ | | 4+ | 4+ | 4+ |
| C-648 | | 2+ | | 4+ | AC | AC |
| C760 | 12/12 | 3+ | | 4+ | 4+ | 4+ |
| MCE-90 | 12/15 | 3+ | 12/21 | 3+ | 3+ | 4+ |
| MCE-92 | | 3+ | | 4+ | 2+ | 4+ |
| MCE-124 | | 2+ | | 4+ | 2+ | 4+ |
| JT-110 | 12/14 | 3+ | | 4+ | 4+ | 4+ |

this animal has been diluted with normal bovine serum to obtain a titer comparable to the pooled positive serum used in Beltsville. One other constant has been added to the test at the suggestion of the Beltsville workers which has further increased the accuracy and comparative test results. This concerns the time interval after inactivation of the serum sample. It was found by the Beltsville workers that maximum efficiency of test is obtained when this interval is 90 minutes. When all of these things had been adjusted, comparative results were considered satisfactory.

At present we believe that we are duplicating the test procedure as conducted by ADPR except for one minor detail. Because guinea pigs must be on green feed to yield the best complement, the Beltsville workers find it advantageous to harvest serum in batches of 100 or more pigs at a time in the fall of the year. This serum is frozen and used as needed. In Hawaii, we normally have an abundance of green feed the year round; pigs are therefore bled out. weekly intervals and the week's supply stored in a dry ice chest until used. The pigs are not exsaguinated. Instead, approximately 10 cc. of blood is drawn from the heart. This volume is replaced with 10 cc of normal saline solution injected intraperitoneally. The pigs are than returned to the colony. The donor pigs are marked by clipping the hair off the neck. When growth of coat removes the distinguishing clip mark, in about 6 to 8 weeks, the animals are again ready for use.

False Positives. Since no biological test is 100 percent accurate, false positives and probably false negatives can be expected. We have no evidence to offer in regard to false negative reactions but do want to place in the record an example of what we consider false positive (Table 15). Subinoculations from KS-87 were made on 2/9/55 and on 8/14/56. Both test calves were negative at the expiration of the 60-day observation period.

We have one other table to present , Table 16. This information was accumulated in the course of preparing this report. No interpretation of these results has been attempted.

A calf trial currently under observation is of special interest:

| | Complement-Fixation | | Test Results | |
|---|---|---|---|---|
| Source of Inoculum | Hawaii | Wash. 4/57 | Hawaii | Wash. .5/57 |
| Waialae #3364 (6 mo. Hol. heifer) | 4/(1:5) | 4/(1:5) | Neg. | Neg. |

Trial calf No. 36 was inoculated intravenously on May 3 with 50 cc. of whole citrated blood from Waialae reactor #3364. Figure I shows developments. On May 29, 1957, 50 cc. of citrated whole blood from calf No. 36 was inoculated into trial calf No. 38. Observations on calf No. 38 to date of this report were negative.

TABLE 15

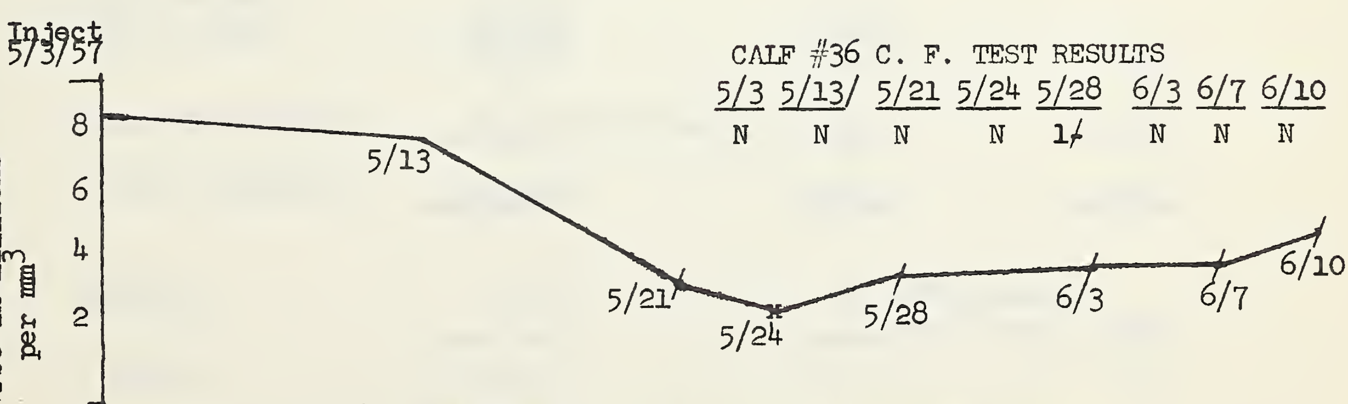
| Ident. | 12/54 Wash. | Redrawn Hawaii | 2/55 Wash. | Herd Retest #1 Haw.11/55 | Herd Ret.#2 Hawaii | 6/56 Wash. | Redrawn Hawaii | 7/56 Wash. | Redrawn Hawaii | 8/56 Wash. |
|---|---|---|---|---|---|---|---|---|---|---|
| KS-87 | 4+ | N | N | N | 4+ | 4+ | N | N | N | N |

| | Redrawn Hawaii | 3/57 Wash. | Retest #3-5/57 Hawaii | Wash. |
|---|---|---|---|---|
| (cont'd) | N | N | N | N |

TABLE 16
SUMMARY OF RESULTS OF TESTING SAMPLES
COLLECTED AT SLAUGHTER FROM TEST POSITIVE REACTORS

| Fiscal Period | | No. Reactors Slaughtered | Slaughter Sample Results No. Pos. | No. Susp. | No. Neg. | Other |
|---|---|---|---|---|---|---|
| Dairy: | '55-56 | 159 | 95 | 32 | 25 | 4 no record |
| | '56-57 | 44 | 25 | 6 | 10 | 1 died in pen<br>1 A.C.<br>1 no record |
| Beef: | '55-56 | 16 | 9 | 2 | 5 | |
| Imp.: | '56-57 | 3 | 1 | - | 1 | 1 no record |
| | TOTALS: | 222 | 130 | 40 | 41 | 8 |

Figure 1




/ - less than) .5% cells with anaplasma bodies
x - more than)

TABLE 17
SUBINOCULATION CALF TRIAL RESULTS
(Other than cattle in Original Quarantine Zone)

| Source of Inoculum | Marginal Bodies Yes | Marginal Bodies No | Marginal Bodies Very Few | C.F. Test Pos. | C.F. Test Neg. |
|---|---|---|---|---|---|
| 13 Individual 4+ Reactors into 13 calves | 9 | 3 | 1 | 9 | 4 |
| 3 Pairs of 4+ Reactors into 3 calves | 1 | 2 | - | 1 | 2 |
| 14 Individual animals. Each had at least one 4+ reaction in either the Wash. or Haw'n laboratory. | 5 | 7 | 2 | 5 | *9 |
| 1 Pair of 3+ suspects into 1 calf | - | 1 | - | - | 1 |
| 11 Groups of animals composited into a single calf. Each group contained at least one 4+ reactor. | 5 | 6 | - | 5 | 6 |
| 1 3+ suspect into 1 calf | - | 1 | - | - | 1 |
| 43 Calf Trials Conducted | 20 | 20 | 3 | 20 | 23 |

(*Note: One sample gave a 1+ reaction)

TABLE 18
SUMMARY OF COMPLEMENT-FIXATION TESTS CONDUCTED 6/1/54-5/31/57

| | Total No. Tests | | Total No. Reactors | % Reactors |
|---|---|---|---|---|
| DAIRY ANIMALS | 36,475 | | 432 | |
| BEEF ANIMALS | 19,842 | | 42 | |
| | 56,317 | | 474 | .84 |
| SLAUGHTER SAMPLES | 85,296 | (7.5% dairy) | 134 | .16 |
| TOTAL TERRITORY: | 141,613 | | 608 | .43 |
| IMPORTS | 5,528 | | 29 (19 before imp. regs.) | .52 |
| TOTALS: | 147,141 | | 637 | .43 |

RECOMMENDATION: In consequence of the problems we have encountered in test technique and precedure, we agree with and concur in the recommendation made by Dr. Mott to Dr. Anderson after a review of our problem last year. There should be central production and distribution of all critical reagents used in the test including antigen, complement, amboceptor and positive and negative sera.

CONCLUSION: The eradication program in Hawaii is essentially a field trial to evaluate the accuracy of the complement-fixation test for the detection of carriers of anaplasmosis using the antigen developed by the workers at Beltsville, now ADPR Division of the Agricultural Research Service.

It is our opinion that our results indicate that the test is sufficiently accurate to serve as a tool for the eradication of the disease under Hawaiian conditions.

# TREATMENT OF ANAPLASMOSIS CARRIERS

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Time will not permit this paper to be given in detail as prepared. However, this report will be compiled with the others and distributed to participants attending this conference.

Since anaplasmosis was recognized as a specific disease by Theiler, 1910, considerable research has been devoted to find a medicinal or medicinals which would be effective against the anaplasmosis-causative agent. Arsenicals, antimalarials, penicillin, streptomycin and chloromycetin have been used. To date none of these have proved effective in the treatment of anaplasmosis.

## CHLORTETRACYCLINE

Foote[2], 1951, demonstrated that chlortetracycline (aureomycin) had anaplasma-inhibitory activity. This finding was substantiated by Miller[3], 1952. The antibiotic was administered intravenously 5 mg./lb./day for four successive days in eight calves and one day in eight calves. Foote[4], 1952, demonstrated that the anaplasmosis-carrier state in a bovine could be eliminated with successive massive doses of chlortetracycline. Three cows were employed in these studies. Two of the cows, 411 and 63, were recovered anaplasmosis carriers. Cow 68 suffered an acute attack of anaplasmosis during treatment, therefore her physical condition would not permit the same regimen of treatment given the other two cows.

Cow 411 received a total of 47.5 gm.; cow 63 a total of 35 gm. and cow 68 a total of 27.5 gm. of the antibiotic.

Cows 63 and 68 were subsequently reinoculated with anaplasmosis-infective blood. Clinical or microscopic signs of anaplasmosis did not develop in either cow. However, after cow 63 was splenectomized anaplasmosis developed[5, 6, 8].

Miller[7], 1952, administered chlortetracycline 15 mg./lb./day for sixteen successive days to 2 steer calves. One calf was splenectomized at the end of therapy. The writer administered to each steer 20 cc. of anaplasmosis-infective blood (33.2% anaplasma bodies) 438 days after termination of treatment. The splenectomized steer died with peracute anaplasmosis twelve days following inoculation. The other steer did not manifest signs of anaplasmosis after this inoculation. However, subinoculation studies proved blood taken from this bovine was anaplasmosis infective 40 days after, but not 90 or 111 days after this steer

TABLE I

DOSES OF CHLORTETRACYCLINE ADMINISTERED COWS 411, 63 and 68

| Cow | Weight | 1st Day | Grams I.V. | 2nd Day | Grams I.V. | 3rd Day | Grams I.V. | 6th Day | Grams I.V. |
|---|---|---|---|---|---|---|---|---|---|
| 411 | 1100 lbs. | 11:00 a.m. | 15.0 | 6:00 a.m. | 10.0 | 6:00 a.m. | 7.5 | | |
| | | 8:00 p.m. | 7.5 | 6:00 p.m. | 7.5 | | | | |
| 63 | 700 lbs. | 11:00 a.m. | 12.5 | 6:00 a.m. | 7.5 | 6:00 a.m. | 5.0 | | |
| | | 8:00 p.m. | 5.0 | 6:00 p.m. | 5.0 | | | | |
| 68 | 750 lbs. | | 7.5 | | 5.0 | | 7.5 | | 5.0 |
| | | | | | | | | 8 hrs. later | 2.5 |

TABLE II

DOSES OF CHLORTETRACYCLINE ADMINISTERED SIX COWS

| Animal | Body Weight | Doses of Antibiotic | Days Between Treatment | Doses of Antibiotic | Subinoculation Results |
|---|---|---|---|---|---|
| AH* | 1200 | 5--10gm.--12 hrs. | 33 | 5--10gm.--12 hrs. | Neg. |
| 139* | 600 | 3-- 5gm.--12 hrs. | | None | Pos. |
| 142** | 600 | 5-- 5gm.--12 hrs. | | None | Pos. |
| McGuire** | 900 | 5--10gm.--12 hrs. | 26 | 5--10gm.--12 hrs. | Pos. |
| 208*** | 700 | 5--7.5gm.--12 hrs. | | None | Pos. |
| Zato*** | 1100 | 3--10gm.--12 hrs. | 22 | 3--10gm.--12 hrs. | Pos. |

*Anaplasmosis carrier
**Convalescent
***Acute Anaplasmosis

was inoculated with the infective blood. Apparently the physiological elimination of introduced anaplasmosis-infective blood resulted in the non-carrier state of this animal[8].

Brock et al.[9], 1953, administered successive massive doses of chlortetracycline to six anaplasmosis-infected cattle, two in the carrier state, two in convalescence, and two in the acute stage of anaplasmosis.

Apparently these large dosages of chlortetracycline had little if any inhibitory effect on the anaplasmosis etiologic agent in animals 139 or 142.

## OXYTETRACYCLINE

Miller et al.[3], 1952, reported that oxytetracycline (terramycin) exhibited definite anaplasma inhibitory properties. The antibiotic was used at the rate of 2 mg./lb./day intravenously. Four calves were treated for three days and twelve calves were treated for one day.

Splitter and Miller[7] reported, 1953, that oxytetracycline, 5 mg./lb./day for twelve and fourteen successive days in single or divided doses apparently eliminated the carrier state of six animals. Subinoculation studies ranging from 30 to 365 days proved the blood from these animals was not infective. One animal was splenectomized at the end of therapy and reinoculated with virulent anaplasma blood 365 days following therapy. The animal developed acute anaplasmosis eighteen days after inoculation.

## TETRACYCLINE

Brock et al.[10], 1955, demonstrated that tetracycline (polyotic) possessed a depressant action on the anaplasmosis etiologic agent. Thirteen calves were employed in this study and were divided into three groups of four calves each and the control group of five calves. One group received one dose of tetracycline intravenously, 3 mg./lb./day and the other group was administered a single dose of the antibiotic intramuscularly, 3 mg./lb./day.

Foote[8] reported, 1955, that tetracycline eliminated the anaplasmosis carrier state in two animals. Four anaplasmosis eperythrozoonosis carriers were used in this research. The antibiotic was administered 12.5 mg./lb./day. One bovine received one dose of the antibiotic, the second two doses at twelve hour intervals, the third three doses at twelve hour intervals and the fourth four doses at twelve hour intervals. Five to eleven days following the last treatment all four animals were splenectomized. The animals receiving one and two doses of the antibiotic recrudesced first with epertythrozoonosis and then with anaplasmosis. The animals receiving three and four doses respectively did not recrudesce with either disease. Subinoculation studies proved the blood of the latter two animals was not anaplasmosis infective. Both were retained for 120 days after splenectomy.

TABLE III

SUBINOCULATION STUDIES OF TREATED COWS

| Animal | Subinoculation Results Days After First Treatment | Subinoculation Results Days After Second Treatment |
|---|---|---|
| AH | 2 Splenectomized Calves Negative | 6 cows--3 to 263 days--Negative |
| 139 | 3 cows (1 splenectomized) 3 to 137 days--Positive | |
| 142 | 1 splenectomized calf--2 days--Positive<br>1 splenectomized calf--36 days--Positive | |
| McGuire | 1 animal--17 days--Positive | 1 animal--7 days--Negative<br>1 animal--51 days--Positive |
| 208 | 1 animal--16 days--Negative<br>1 animal--74 days--Positive | |
| Zato | 1 heifer--21 days--Negative | 1 animal--4 days--Negative<br>1 animal--46 days--Positive |

Pearson et al.[11], 1957, used tetracycline 5 mg./lb./day in three lots of ten cattle each. Five animals of each lot received the antibiotic intravenously and five intramuscularly. The animals in lot I were treated for five consecutive days; those in lot II were treated for five consecutive days, rested ten days and treated an additional five days and lot III received treatment for ten consecutive days.

Anaplasmosis-carrier infection was eliminated in six of the ten cattle in lot I; nine of the ten cattle in lot II and all ten cattle in lot III. Results obtained in lots I and II indicate that tetracycline, 5 mg./lb./day as used in this study is on the border line of effectiveness when used to eliminate the anaplasmosis carrier state. The authors recommend tetracycline, 5 mg./lb./day intramuscularly for ten consecutive days to eliminate anaplasmosis-carrier infection.

## AUREOFAC-10

Brock et al.[12], 1957, reported the prevention of anaplasmosis in 10 steers fed aureofac-10. Each of the 10 steers were administered 5 ml. of anaplasmosis infective blood one week prior to the aureofac-10 feeding program. Five steers were individually fed 1.0 mg. of chlortetracycline and 5 steers were individually fed 0.5 mg. chlortetracycline (as aureofac-10) per pound body weight daily in feed concentrate for 60 days. None of these steers was carriers of anaplasmosis at the end of treatment.

Splitter et al.[13], 1957, reported that the anaplasmosis carrier state in the bovine could be eliminated by placing aureofac-10 in the feed. Seven animals received chlortetracycline, a total of 5 mg./lb./day (as aureofac-10) placed in the feed two times daily for 30-75 days. Three animals received chlortetracycline, a total of 10 mg./lb./day (as aureofac-10) placed in the feed two times daily for 14-45 days. The anaplasmosis carrier state was eliminated in all ten of these animals. However 3 animals were fed chlortetracycline, a total of 5 mg./lb./day for 14 days and one was fed chlortetracycline, a total of 10 mg./lb./day (as aureofac-10). The aureofac-10 was placed in the feed two times daily. This regimen of feeding did not eliminate the anaplasmosis-carrier state of these animals.

Six of the 10 animals in which the anaplasmosis carrier state was eliminated by feeding aureofac-10 were reinoculated with anaplasmosis-infective blood. These cattle proved susceptible to the disease.

Pearson et al.[14] reported the feeding of chlortetracycline (as aureofac-10) to 2 animals, 5 mg./lb./day and to 3 animals, 2.5 mg./lb./day for 60 days. The anaplasmosis-carrier state was eliminated in all 5 animals.

The cost of the antibiotics, used intravenously to eliminate anaplasmosis-carrier infection, ranges from 60 to 100 dollars per 1000 pound animal. It is

economically prohibitive to use any of these antibiotics routinely to eliminate anaplasmosis-carrier infection in grade animals.

The cost of eliminating the anaplasmosis-carrier state in a 1000 pound bovine by feeding chlortetracycline 5 mg./lb./day, (as aureofac-10) ranges from 20-30 dollars.

It is reported that animals placed on chlortetracycline, 2.5 to 10 mg./lb./day (as aureofac-10) go off feed for 2-4 days. Such animals apparently then become accustomed to the feed and consume normal quantities.

The complement fixation test certainly offers promise in identifying anaplasmosis-carriers. However, there are some inconsistencies when comparing it with subinoculation studies and also its application in the field. Subinoculation studies of one animal at 100, 200 and 365 days after therapy were negative. The C. F. test of this animal was negative 293 and 365 days after therapy, but positive 394 and 421 days after therapy. However, one animal was positive to the test 7 days after treatment, but was negative to subinoculation studies 33 days after therapy. Tetracycline studies by Pearson et al.[11] revealed that three animals; 38, 71 and 708 were positive to the C. F. test for 76, 189 and 237 days respectively after treatment was started while they remained negative to subinoculation studies for 73, 196 and 237 days respectively. Animal 708 received 10 consecutive treatments of the antibiotic intravenously.

In the dairy herd of the Louisiana Agricultural Experiment Station, Iberia Livestock Dairy Station, Jeanerette, Louisiana, the U.S.D.A.; A.R.S., C. F. test, April 1957 showed 6 animals to be anaplasmosis negative. The December C. F. test, 1956, U.S.D.A.; A.R.S. indicated these same 6 animals to be anaplasmosis-positive. Two of these six cows suffered acute anaplasmosis last summer (1956) and were treated with one dose of 2.5 gm. tetracycline intravenously. This single small dosage has not eliminated the anaplasmosis carrier state experimentally. Bovine eperythrozoonosis is present in this herd and it is possible that the causative agent of this disease prevents the production of the anaplasmosis C. F. antibody.

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# PREVENTIVE THERAPY OF ANAPLASMOSIS BY FEEDING CHLORTETRACYCLINE

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After having done much work with the tetracycline antibiotics in the field of destruction of the carrier state of anaplasmosis, the personnel of the Oklahoma station began investigating the possibility of preventive therapy by feeding chlortetracycline hydrochloride (Aureomycin). After much discussion with others working in the same field, and related fields including production of antibiotics, we decided to first screen the dosage by administration of the chlortetracycline orally in capsules.

The procedure was as follows. The animals would be injected with 5 cc of carrier blood. One week later, the feeding of chlortetracycline of Aureofac 10 origin would start and would be continued for sixty days. At this time, a sample of at least 100 cc of blood would be taken and injected into a splenectomized calf. The animal would be held sixty more days and another sample of blood would be drawn and injected into another splenectomized calf. During the sixty day feeding period, samples would be drawn frequently for red cell counts, hemoglobin, hematocrit and stained slides. Weekly samples would be drawn for complement-fixation test during the entire 120 days.

Six splenectomized calves were used in the first screening procedure. All were given five cc of blood from a carrier animal.

Number 411 received .5 mg per pound of body weight of chlortetracycline daily.

Number 412 received 1 mg per pound of body weight of chlortetracycline daily.
Number 460 received 2 mg per pound of body weight of chlortetracycline daily.
Number 461 received 3 mg per pound of body weight of chlortetracycline daily.

The two controls received the same feed but no chlortetracycline and developed normal cases of anaplasmosis. All four of the treated animals remained normal and negative to anaplasmosis throughout the experiment as did all the calves injected from them at the 60 and 120 day intervals. All the complement-fixation reactions on the treated animals remained normal.

With the screening at the 0.5 mg to 3 mg per pound of body weight daily completed, we obtained 15 yearling Hereford steers weighing slightly over 800 pounds each. These were divided into three lots of five each. All were inoculated with five cc of blood from a carrier animal.

The animals in lot one received 1 mg. chlortetracycline daily per pound of body weight. Lot two received 0.5 mg per pound of body weight daily and lot three received no treatment.

The foregoing treatment was given in feed and each animal received one pound of feed for each 100 pounds of body weight. However, all the animals in such lot were fed in single troughs as in any feed lot.

The control cattle became infected with anaplasmosis, and were positive to the cf test. The treated animals all remained negative to the cf test except one, which showed a four plus reaction on the 28th day, but became negative by the 34th day following inoculation. All remained negative on the blood smears, and all of the splenectomized calves inoculated at the 120th day following the start of feeding remained negative.

To further determine the lowest possible level of dosage of chlortetracycline, four splenectomized calves were injected with five cc of blood from a carrier animal. One was used as a control and the three remaining received 1/10 mg. per pound of body weight per pound per day orally in capsules. Several animals were put on the same dose, as this dose approximates that used in feed lot experiments for increased weight gains.

The control animal became positive and one animal, #566, died from bloat on the 42nd day of treatment. Five cc of blood was obtained on post mortem and inoculated into a splenectomized calf which became infected with anaplasmosis.

The two animals that completed the screening remained negative on stained slides, and to the cf test during the treatment. Number 560 became positive to the cf test seventeen days after being taken off treatment and positive on the stained slide twenty-three days after the completion of treatment. Number 561 became positive on the stained slide forty-four days after treatment was completed. The calves sub-inoculated with 150 cc of blood from the treated animals at the completion of treatment, did not become positive.

With the screening completed on the 1/10 mg per pound of body weight, we obtained twenty yearling Hereford heifers weighing slightly over 500 pounds each. They were divided into four groups of five each. Two groups were used for controls and two for treatment.

On this experiment there were some changes in procedure. All twenty animals were inoculated with ten cc of blood from an active case of anaplasmosis. The time between inoculation and the start of treatment was nine days instead of seven, and the animals received the chlortetracycline in three pounds of feed daily instead of one pound per 100 pounds of body weight. The chlortetracycline was fed for 90 days.

The animals were also fed in groups of five as before, not individually. All the ten control animals developed anaplasmosis with a very short incubation period. All were positive to the cf test and on stained slides on the fifteenth day following inoculation. All animals in the two groups on treatment also became positive to the cf test on the fifteenth day, and remained so throughout the experiment. At that time, as well as at later intervals, some anaplasma bodies were visible on the stained slides. It should be noted that while these animals contracted the disease, there were few visible bodies on the stained slides and the hemoglobin, hematocrit and red cell count remained normal throughout the experiment while the controls developed normal cases of anaplasmosis for cattle their age. Also the treated animals did not show visible symptoms of the disease.

Although the disease was not prevented and the animals became carriers of the disease, this level of treatment has some promise of value in feed lot cattle that are going to be slaughtered. To be able to give you an early report and support the positive cf test, a pooled sample of blood was injected into a calf which became infected with anaplasmosis. The time for final individual inoculations is not completed.

## SUMMARY

On the screening procedure in this series of experiments, ten splenectomized calves were used. Seven on treatment and three for controls. The dose range of chlortetracycline varied from 1/10 mg per pound of body weight to three mg per pound of body weight. All levels from 0.5 mg up prevented anaplasmosis from developing, but 1/10 mg per pound of body weight was not sufficient to do so.

In the experiments proper, three levels of chlortetracycline were fed. Five head of cattle received 1 mg per pound of body weight a day, five received 0.5 mg per pound of body weight, and ten head received 0.1 mg per pound of body weight. The animals treated for sixty days on 0.5 mg and one mg per pound of body weight did not develop anaplasmosis or the carrier state of the disease. The ten head of cattle on 0.1 mg per pound of body weight for ninety days developed anaplasmosis mildly and the carrier state was present following treatment. Clinical symptoms were absent. This dose level may be of value in feed lot cattle. The twenty control animals for the former three groups all developed anaplasmosis.

# ANAPLASMOSIS RESEARCH ACTIVITIES IN THE UNITED STATES

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The part of this conference assigned to me is a report of research currently in progress on anaplasmosis in the United States. In order to obtain this information an informal formal request was sent out on May 3, 1957. These letters went to the Deans of the Schools, Divisions or Colleges of Veterinary Medicine; to the Agricultural Research Service, United States Department of Agriculture, both research and regulatory and to Heads of Departments of Veterinary Science, Animal Pathology, etc. at institutions not giving the D.V.M. degree. No one was asked how intensively he was pursueing the various objectives or how much money was assigned for this work.

We are therefore indebted to the folks who answered this letter for the information which is compiled in this brief report. To narrate the comments which came would make this report too long so at the outset I am mentioning the states and agencies which indicated that they currently have projects. After this we are summarizing the objectives and listing by each the states which are working in these respective areas.

People do not express themselves in the same way so I have attempted to pull together objectives (which seem to indicate one particular line of research approach or area on the subject) and then listed the states. In most cases the objectives are not exactly as the reporters sent them because of the attempt to consolidate.

In alphabetical order the following states or agencies reported work currently in progress on anaplasmosis: University of California, University of Florida, Kansas State College, Louisiana State University, Montana State College, University of Nevada, Oklahoma A. & M. College, Oregon State College, University of Pennsylvania, University of Tennessee, Texas A. & M. College, and Agricultural Research Service, United States Department of Agriculture.

The Director of the Agricultural Experiment Station, University of Hawaii, where they are not doing research on this disease, asked Dr. E. H. Willers, Territorial Veterinarian, to answer the inquiry. As you know a cooperative program is being carried by ARS and the territory of Hawaii designed to eradicate anaplasmosis from that area. Even though this is designed primarily as an eradication program it may prove to be an exceptionally valuable research finding, therefore I am mentioning it and those in attendance can interpret it as research or regulatory activity as they like.

One state which for sometime did a considerable amount of work on anaplasmosis is dropping its projects for the time being. However, the person in charge indicated that in his opinion there was certainly room for more work in the areas of: further studies on development of the antigen and use of the complement fixation test; field studies of the test; more work on chemotherapeutic agents with particular reference to eliminating the carrier state; and more work on the problem of vectors and the elimination of vectors.

If you will observe the map, given to you by Dr. Saulmon, as I recite the work being carried on you will see that the primary interest, as it should be, is in the areas which are having the most trouble with anaplasmosis except for Pennsylvania. Unfortunately for research workers this problem is not concentrated in one segment of the country so that the workers can get together at frequent intervals. Rather there are points of concentration in the South and Southeast and in the Southwest and south central part of the country, then a big jump to the West coast and the Northwest, Rocky Mountain, and Intermountain areas.

The most intensive work areas of research are as follows:

a) studies on the nature of the etiologic agent,
b) studies on the complement fixation test, and
c) studies on the use of antibiotics.

a) California and the U.S.D.A. are concerned with pathogenesis or infectivity; California, Louisiana and Oklahoma are concerned with the pathology produced. Cultivation of the agent, embryo inoculation, tissue culture and in vitro studies are being conducted by Florida and Pennsylvania. Adaptation of the agent to unnatural hosts is being attempted in Louisiana. Texas and Louisiana also are studying the nature of the causative agent.

b) The complement fixation test is being studied from several angles by several groups. Detailed study of the test as such is in progress in Florida. It is being used on a survey basis with research as well as eradication programs as objectives in Kansas and Montana. The use of and evaluation of the cf test as a means of control are specific objectives at Oklahoma, Oregon and Texas. The test is being combined with herd management programs in Tennessee. The specificity of the test when eperythrozoonosis is a factor is a joint project of Louisiana and the U.S.D.A. Field incidence surveys and field eradication studies as well as continuing field studies with the test are being conducted by the ARS. Purification of the antigen and development of a sensitive, specific serological test is being worked on by Pennsylvania.

c) The effectiveness of wide spectrum antibiotics is being studied intensively. Control of the Infection is a specific objective in California, Louisiana and Oklahoma and the use of antibiotics for the eradication of the infection is in progress in Kansas and Oklahoma.

Immunity studies of several kinds are objectives of the ARS and the states of Louisiana, Nevada and Oklahoma. The vector problem is being studied by the ARS, Oregon and Louisiana. Infectivity and transmission studies are being made by the ARS and Oregon. Clinical observations and studies as related to complement fixation and other diagnostic procedures as well as the relationship to vectors is a major consideration in California. Diagnosis by the flourescent technique is a study of Florida.

Still other areas of research reported are as follows: the role of deer in the epidemiology is an objective at California; the interference of eperthrozoonosis in the experimental study of Anaplasmosis, Louisiana; Anaplasmosis in sheep, Kansas; and the electron microscope is being used as a research tool by Texas and Pennsylvania.

The University of Pennsylvania School of Veterinary Medicine is conducting its' work on Anaplasmosis at the laboratory in Palo Alto, Federal District, Mexico under the direction of Dr. Carlos Espana who tomorrow will discuss "Electron Microscopy in Anaplasmosis".

The material received was too voluminous to put on a chart. For that reason it has been given in a quite abbreviated narrative. Attached is a resume of objectives as reported and the name of the reporter. It is believed this will be helpful as a reference to those who would like to contact others doing work on this problem.

## Anaplasmosis Research Objectives by Agencies and Reporter

California--Reporter: Dr. J. F. Christensen

1. Studies on the Nature of the Etiological Agent of Anaplasmosis.
2. Control of Infection by the Use of Wide-Spectrum Antibiotics.
3. Clinical Studies.
4. The Role of the Deer in the Epidemiology of Bovine Anaplasmosis.

Florida--Reporter: Dr. D. A. Sanders

1. Nature of the Causative Agent--Chemical and Physical Properties, etc.
2. Diagnosis by Fluorescent Technique.
3. Propogation by Tissue Culture.
4. Embryo Inoculation Studies.
5. Complement-Fixation Studies.
6. Electron Microscopy Studies.
7. Fluorescent Technique and Intermediate Host Studies.

Kansas--Reporter: Dr. M. J. Twiehaus

1. Antibiotics in Eradication of Infection from Carriers.

2. Complement Fixation Test Survey.
3. Anaplasmosis in Sheep

Louisiana--Reporter: W. T. Oglesby

1. Studies on the Nature of the Etiological Agent.
2. Studies on Vaccines.
3. Adaptation of the Etiological Agent to an Unnatural Host.
4. Periodically Work With Newly Released Medicinals which on the Basis of Action Against Other Diseases Might be Helpful.
5. Study of the Eperythrozoonosis-Anaplasmosis Complex because the Former Interferes Materially with Anaplasmosis Research.
6. With Agricultural Research Service, Study Complement Fixation Test and its Specificity where Eperythrozoonosis is Concerned.
7. By Use of Radioisotopes Study Red Blood Cell Formation and Destruction. (With Animal Industry)
8. Vectors--by Department of Entomology

Montana--Reporter: Dr. E. A. Tunnicliff

1. Continuation of the Complement-Fixation Survey of the Incidence of the Disease.
2. "Our Main Objectives are Eradication and a Survey of the Incidence of the Disease."

Nevada--Reporter: Dr. Kenneth L. Kuttler

1. To Develop a Method of Immunization in Cattle by the Use of Inactivated Tissue Vaccine.

Oklahoma--Reporter: Dr. Glenn C. Holm

1. "Immunization and Treatment of Cattle with Anaplasmosis". Various Immunization Studies and Treatment with Antibiotics.
2. Evaluation of the Complement-Fixation Test as a Means Toward Control.
3. Pathology of Anaplasmosis.

Oregon--Reporter: Dr. E. M. Dickinson

1. Epizoology of Anaplasmosis in Different Selected Herds by Annual Complement Fixation Tests of Breeder Animals and Calves.
2. Transmission Studies and Possible Reservoirs of Infection. Splenectomized Calves will be Test Animals.
3. Study of Athropod Vectors in Co-operation with Entomologists.

Pennsylvania--Reporter: Dr. M. W. Allam

1. Cultivation of Causative Agent-Study of the Agent in Vitro.
2. Studies on the Nature of the Causative Agent.
3. Purification of Antigens and Development of "A Sensitive Specific Serological Test."

Tennessee--Reporter: Dr. G. N. Merriman

1. Determine the Efficiency of Three Methods of Controlling Anaplasmosis
   (a) Test and Slaughter of Reactors
   (b) Test and Retension of Reactors in Isolated Herd on Same Premises as Non-Infected Cattle.
   (c) Test with Retension of Reactors in Same Herd as Non-Infected Cattle.
2. Determine a Calf Inoculation Test Accuracy of Complement Fixation Test Under <u>Our</u> Conditions.

Texas--Reporter: Dr. T. E. Franklin

1. To Determine the Characteristics of the Causative Agent of Anaplasmosis.
2. To Develop and Evaluate Serological Testing Procedures for the Detection of Anaplasmosis in Animals.
3. To Develop Effective Treatment for Anaplasmosis

Agricultural Research Service, U.S.D.A.--Reporter: Dr. Lawrence Mott

1. Diagnosis--Continued Study in the Field of the C.F. Test Developed by This Agency.
2. Training of Technicians. This may not be considered research by some but it is a most important part of the over-all program.
3. Studies of Anaplasmosis in the Tick.
4. Infectivity and Transmission Studies (A complex program but promising very valuable results.
5. Immunity Studies--This is quite broad from the standpoint of studying possible passive immunity and attempting to develop immunological agents.
6. Field Incidence Surveys and Field Trial Eradication Studies. These are based on using the C.F. test to find reactor animals. Some work is in cooperation with groups inside U.S.D.A.

# ELECTRON MICROSCOPY IN ANAPLASMOSIS*

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## Introduction

One of the most important problems to be solved in the study of bovine anaplasmosis is that of the nature of the etiological agent. Although most workers in this field seem to agree that Anaplasma marginale is a parasite belonging to the protozoa, no definite evidence has been presented to show that there is a developmental cycle. Moreover, nobody has been able to determine the exact mode of reproduction of this parasite nor has it been possible to cultivate A marginale in vitro. De - Robertis and Epstein (1) in a study of the parasite with the electron microscope were able to demonstrate that the marginal bodies within the erythrocytes of animals affected with anaplasmosis, possess certain internal structure. This finding, if confirmed, would be helpful in explaining some of the clinical features of the disease as well as the probable stages in the multiplication cycle of the anaplasma.

The purpose of the present work was to confirm De Robertis and Epstein's findings since it was considered of theoretical as well as practical importance to determine where A. marginale is composed of submicroscopic units not revealed by the optical microscope, as shown by these investigators.

Before describing the results obtained, I must emphasize that this study is of a preliminary nature and should be considered as a progress report rather than a complete investigation. The only reason why we are willing to present preliminary results at this time is that we feel it is important to report that we have been able to confirm, in part at least, the findings of the above workers, which as far as we know has not been done as yet.

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## Materials and Methods

Source of Infective Blood. -The strain of *A. marginale* used in these studies was originally obtained from a bovine carrier 12 days after splenectomy and has since been passed serially through susceptible bovines not splenectomized. Most of the experiments to be reported were carried out with blood drawn during the acute stage of the disease, representing the 4th serial passage of the strain.

Electron Microscopy. -A Philips 100 kV. electron microscope was used in these studies.* The magnification generally employed was of 11, 000 to 15, 000 X.

Several methods of preparing the blood for electron microscopy were tried. At first we used the conventional method of making a dilute suspension of saline washed red cells and mounting them on metallic grids covered with a collodion film. This procedure was unsatisfactory in most instances. The parlodion replica technic developed by Epstein, Fonseca and De Robertis (2) in which blood smears was used met with no success in our hands.

Recently a method was developed whereby satisfactory preparations could be obtained more regularly. It consists of freezing small amounts of whole blood, either defibrinated or collected in Alsever's solution, in sealed ampules at -20°C. Prior to use, the blood is thawed rapidly at 37°C and centrifuged in the multispeed attachment of a refrigerated centrifuge at 10, 000 rpm for 10 minutes. The supernatant fluid is discarded and the sediment washed 2 or 3 times in distilled water or until the supernatant fluid is free of hemoglobin. After the last washing the sediment is resuspended in an amount of distilled water equivalent to twice the original volume of blood. By means of a micropipette, drops of this suspension are transferred to standard nickel grids with collodion film supports. The stromas are allowed to settle for 5 minutes and the excess fluid removed The films are left to dry at room temperature and examined directly or after light shadowing with palladium at arc tangent 2/10.

Results. -Figures 1 and 2 show infected erythrocytes from a bovine with acute anaplasmosis at a time when the number of parasites was beginning to increase rapidly. The anaplasmas appear as masses of high electron density measuring between 0.8 to 1.0 micron in diameter. It will be noticed that the parasites possess an internal structure consisting of rounded bodies surrounding a central portion of lower electron density. We were not able to find at this stage of the infection submicroscopic units dispersed throughout the infected erythrocyte as were shown by De Robertis and Epstein. It is possible that most of the parasites observed were at the beginning of their developmental cycle prior to the phase of multiple division.

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* I should like to express my appreciation to Phillips S E T de Mexico for kindly extending to us the use of their facilities for electron microscopy.

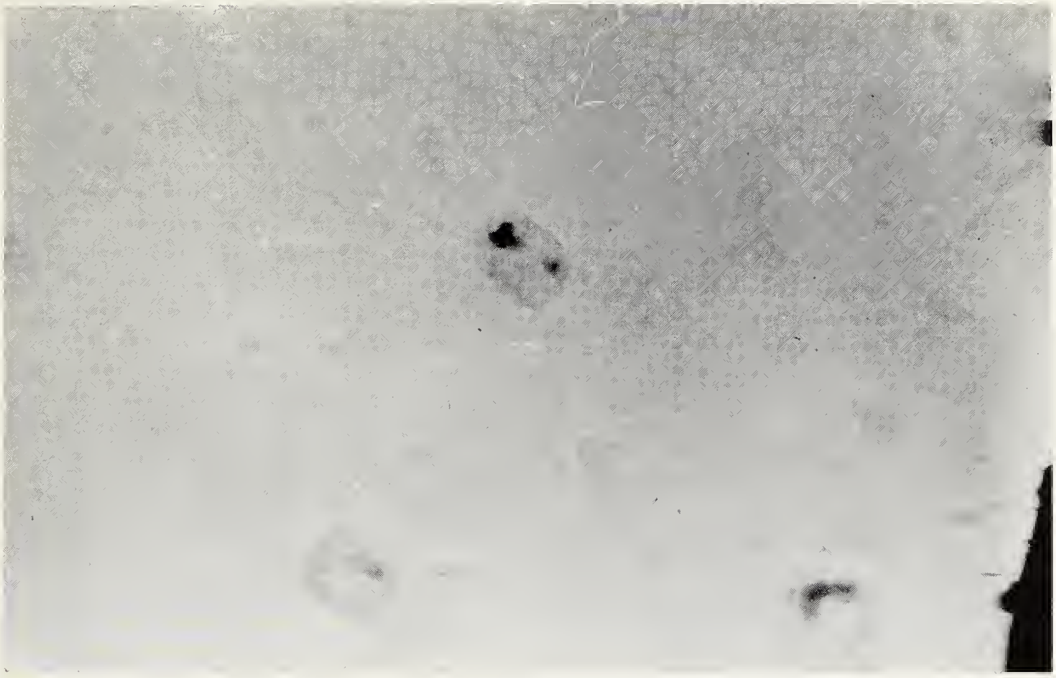

Fig. 1 Red cell membranes of a non-splenectomized bull infected with *A. marginale*. 12,000 X shadowed with palladium at the angle tangent 2/10.

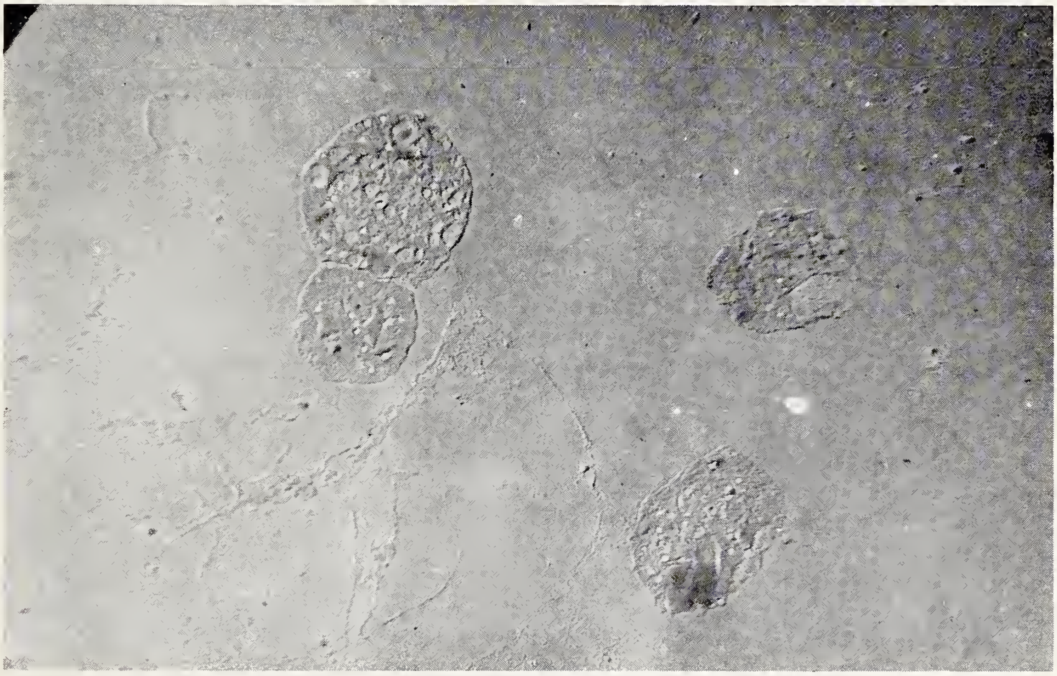

Fig. 2 Red cell membranes of a non-splenectomized bull infected with *A. marginale*. 12,000 X shadowed with palladium at the angle tangent 2/10.

Several infected erythrocytes are illustrated in figure 3. The blood was taken at the height of the disease when there were about 40 percent of red cells parasitized. In addition to the bodies present in the mass of the parasite, one sees very small round bodies dispersed within the red cell. It is very difficult with the available information to make an interpretation regarding the nature of these structures. Before we can draw any definite conclusions as to whether or not they form a part in the developmental cycle of A. marginale, it will be necessary to prove that they are always present in infected and never in normal bovine erythrocytes. Work is still in progress to clear this point.

Figures 4 and 5 show infected erythrocytes from a splenectomized calf during the acute stage of the disease. It will be seen that the parasites present in each case the general pattern previously described. It should also be mentioned that several anaplasmas were found with no evidence of internal structure, which might represent immature parasites. This would confirm in part the observations of Lotze and Yiengst (3). These workers treated blood smears from infected animals with 0.5% acetic acid and found that small anaplasmas were devoid of internal structure, possessed smooth outline, and underwent growth followed by multiple division. On the other hand, we have so far been unable to find under the electron microscope the extra-erythrocytic forms described by Lotze and Yiengst.

Summarizing, we feel that the similarity of our preliminary findings with those of De Robertis and Epstein seems to indicate that the single marginal bodies as revealed by the optical microscope actually consist of a central undivided mass surrounded by elementary-like bodies of high electron density and of a size below the limit of resolution of the ordinary microscope. This internal structure would suggest that these submicroscopic units may represent a phase in the developmental cycle of A. marginale within the red cell. It is significant to point out that we observed very similar structures to those seen by the above workers even though a completely different technic was used. This would seem to rule out the probability that what we observed were artifacts.

## Discussion

In the light of available information it would seem that the marginal bodies observed within the erythrocytes of bovines with anaplasmosis represent parasites capable of growth followed by multiple division. However, none of the evidence presented in the literature thus far, including electron microscopy and histochemical reactions (4), has revealed the exact nature of the parasite, the complete developmental cycle nor the precise manner in which A. marginale invades the erythrocyte. Furthermore, nothing is known about the presence of exoerythrocytic forms which would be very helpful in explaining the clinical and hematological evolution of the disease.

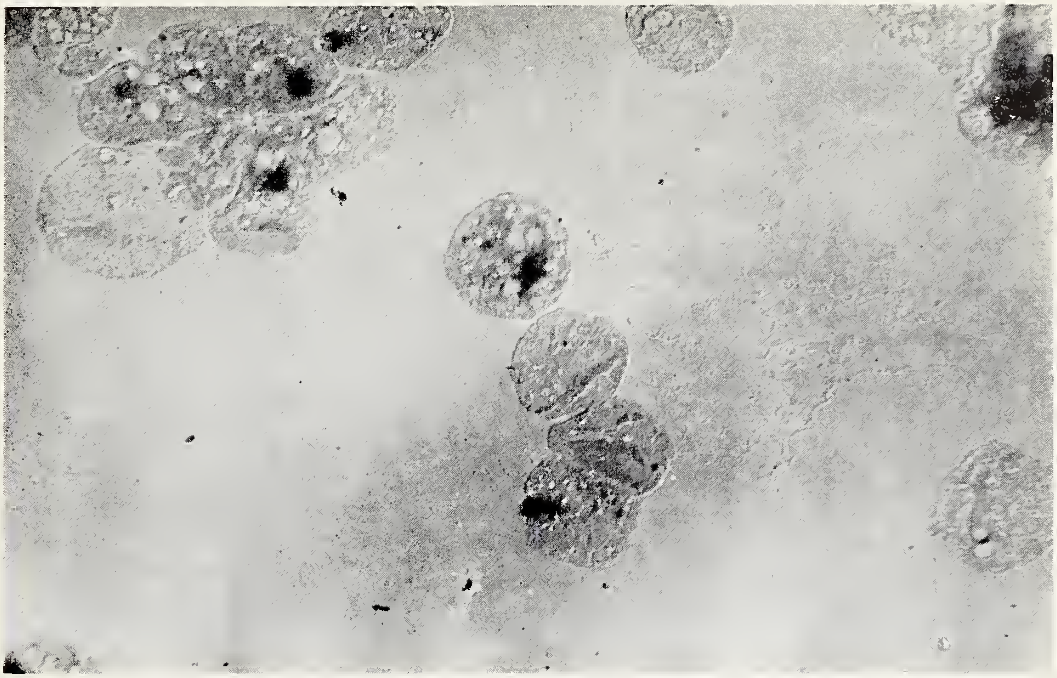

Fig. 3 Red cell membranes of a non-splenectomized bull infected with *A. marginale.* 12,000 X shadowed with palladium at the angle tangent 2/10.

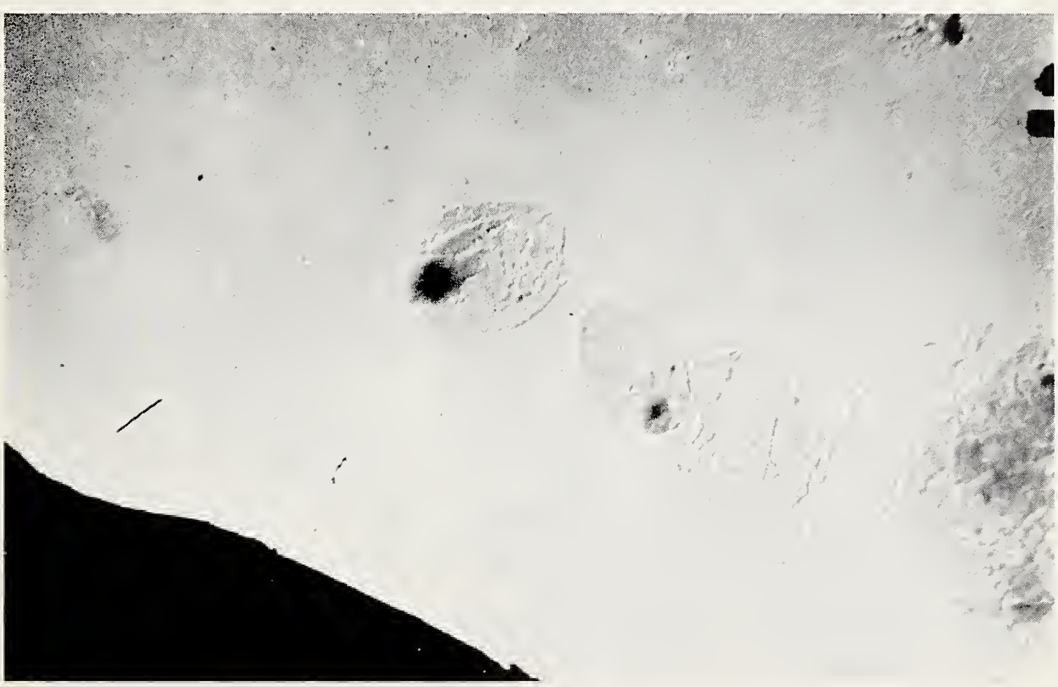

Fig. 4 Red cell membranes of a splenectomized calf infected with *A. marginale.* 11,-000 X shadowed with palladium at the angle tangent 2/10.

Fig. 5 Red cell membranes of a splenectomized calf infected with *A. marginale*. 11,-000 X shadowed with palladium at the angle tangent 2/10.

## Summary

*Anaplasma marginale* was studied under the electron microscope. Our preliminary results seem to confirm previous observations that the parasite in the mature stage is composed of a central undivided mass and peripheral rounded bodies of high electron density of a size below the limit of resolution of the optical microscope. The occurence of these submicroscopic units may represent stages in the developmental cycle of this parasite within the red cell and also help to explain some of the clinical features of the disease, such as the persistence of the infective agent in the blood of carriers in which very often typical anaplasmas cannot be found or recognized with certainty.

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# EPIZOOLOGY OF ANAPLASMOSIS IN THE NORTHWEST

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The story of anaplasmosis is an old one to those who live in the Southeastern states. In fact, anaplasmosis may have been imported with the first Spanish cattle and remained obscure until recognized academically in 1913[1] and as a serious disease of bovines in 1925.[2] As late as 1939, it was classified as a "tropical or semi-tropical" disease.[3] Simms has suggested that the quarantine of southern cattle because of piroplasmosis contained anaplasmosis in the southern states until the lifting of that barrier.[4] Suffice to recognize that in relatively recent years the infection has spread westward and northward until few if any states have remained anaplasmosis free, and the intermountain area of the Northwest has become what must be considered a seriously infected area.

In discussing the epizoology of anaplasmosis in the Northwest, it should be emphasized that, as might be expected, the borders of the principal infected area are ecological rather than geographical as will be pointed out, and that in discussing the problem a fairly well defined area including the whole or parts of each of the eleven western states must be considered as a unit.

Recognized in California in 1925[2] and in Nevada in 1927,[5] the disease probably spread northward and was diagnosed in Oregon 1935,[6] Idaho 1937,[7] Montana 1943,[8] and Washington 1952.[9] The other intermountain states also recognized the disease during the same period, and if the progression of the disease in Oregon is a fair example, spread is continuing in all of these states.

While these dates of recognition are probably not absolute criteria of the rate of spread, they do indicate a progressive northward expansion of the epizootic area. It is interesting to note that this spread of anaplasmosis has not been as rapid as in the case of the more highly contagious diseases. One might expect that with the first movement of carriers into a clean area, widespread infection would result. This apparently has not been the case, even in the presence of considerable numbers of potential vectors. It appears that a few hundred years may have elapsed between the time the first infected Spanish cattle entered Florida and the time when the disease was recently experienced in the state of Washington.

Survey testing of some 3,500 animals in the epizootic area of Oregon has revealed that in some instances infection, based on positive reaction to

the complement-fixation test, approaches 100 per cent. This presents a real problem when a control program is considered, and a somewhat different research problem than exists in the Southeast. Since in many instances most females have been exposed by the time they reach maturity, clinical cases with resulting loss occurs mostly in bulls purchased from areas and turned on the infected range.

The complexity of transmission (and spread) of anaplasmosis, and the incompleteness of our knowledge concerning those factors has been emphasized by Dikmans.[10] Although much has been added to our information since that publication, the apparent slow rate of spread of the disease into the Northwest cannot be satisfactorily explained. This slow rate of spread does suggest, however, that perhaps a considerable reservoir of infection must obtain in an area before the disease becomes economically important.

This reservoir of infection has not been well explored. Boynton[11, 12] demonstrated that the two species of deer common to the Northwest are natural carriers. The status of the pronghorn and elk both common to much of the region is unknown. Likewise the importance of some 22 genera comprising some 40 species of rodents and rabbits common to the region is quite unknown. They do of course serve as hosts for some indigenous ticks, and these ticks in turn no doubt constitute the most important portion of the reservoir.

As late as 1934 DuToit wrote "In nature anaplasmosis is transmitted exclusively by ticks."[13] In the light of more recent research findings other arthropods must be considered as potential vectors;[10] however, ticks are still recognized as the most important vectors in some regions, and this is apparently true of much of the intermountain country of the Northwest. It is true that there are some "off season" cases of anaplasmosis in the region, and some of these may result from occasional transmission by other arthropods. It must be also kept in mind that while the activities of ticks are generally quite seasonal, these activities are governed by temperatures and moisture conditions. At high elevations active ticks can be found throughout the summer months and well into the fall.

That flies play only a minor role in some of the region is indicated in the results of field investigations carried out in an area of relatively high tabanid population. In this area (south central Oregon), there is a decline in the number of clinical cases occuring not long after emergence of the flies with very few cases occurring after July 1. If tabanids were important vectors in this area, a wave of infection should follow sometime after emergence of the flies, but this is not the case.

Mosquitoes are abundant in part of the region, being associated with marshy areas in some instances and with the melting of snow and the resulting pools in others. Their role as vectors need further exploration.

Four species of Dermacentor ticks proven experimentally as anaplasmosis vectors (10) are found in the region.(14, 15) Where present, these four species must all be considered potential vectors. More complete tick surveys and additional experimental work may reveal others.

D. occidentalis, a multiple host tick, is relatively common on the south coastal slope but apparently diminishes in numbers in the northern coastal slope. Anaplasmosis has not yet been diagnosed in the coastal slope north of 44°.

D. variabilis, quite common in California, has been collected in a few scattered areas in the Northwest. It cannot at this time be considered an important vector in that area because of its limited occurrence, although it is a multiple host tick.

D. albipictus, a one-host tick, is widely but intermittently distributed in the eleven western states. Also known as the "winter tick", it cannot be recognized as a principal vector since only a relatively few cases of anaplasmosis have occurred during that period of the year.

The habitat of D. andersoni involves all or part of the eleven western states and extends into Canada. In several respects this tick fulfills the requirements of an ideal vector: 1) It is abundant throughout much of its habitat and at times the population is tremendous.(16) 2) It is a very adaptable multiple host tick, having been found on more than 50 species of mammals in Oregon alone.(15) 3) Transovarian passage of the etiological agent of anaplasmosis has been demonstrated.(17) 4) It has a long life span and an adult failing to find food the first two seasons may present itself for three consecutive years.(18)

Our studies have shown D. andersoni to be the only tick consistently found on cattle in much of the epizootic sagebrush area in Oregon. The testing of cattle maintained in adjacent irrigated districts which are quite free of ticks has shown them to be relatively free of anaplasmosis. These observations and the seasonal occurrence of anaplasmosis in this area indicate that this tick is probably the principal vector.

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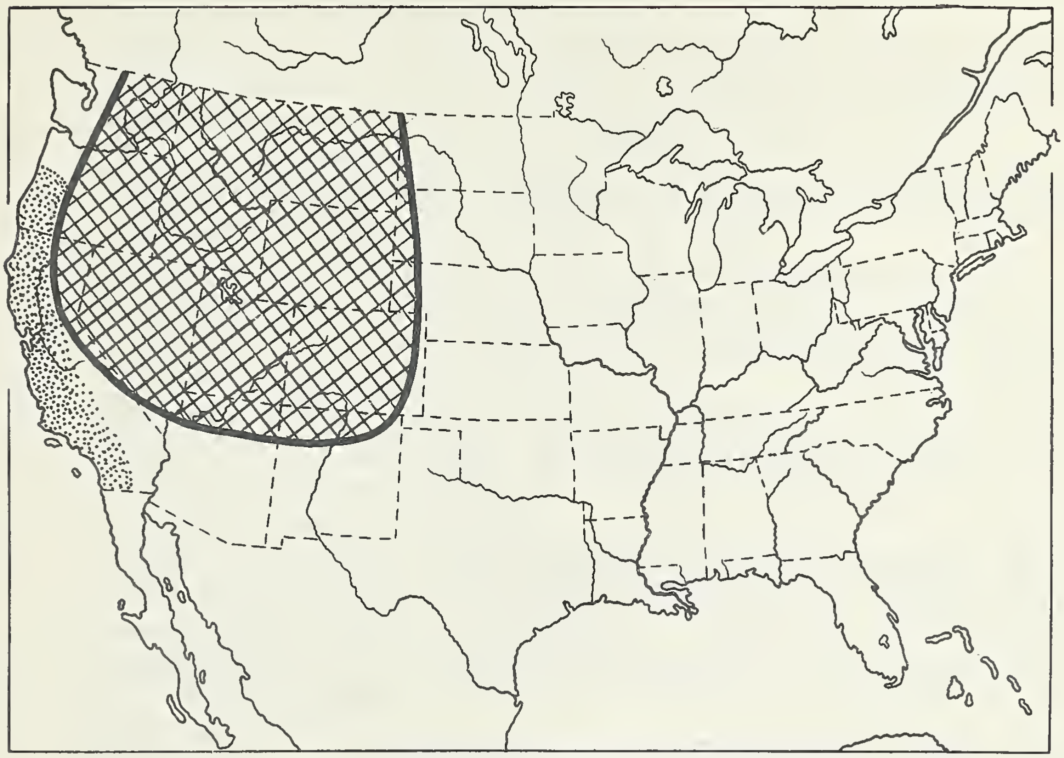
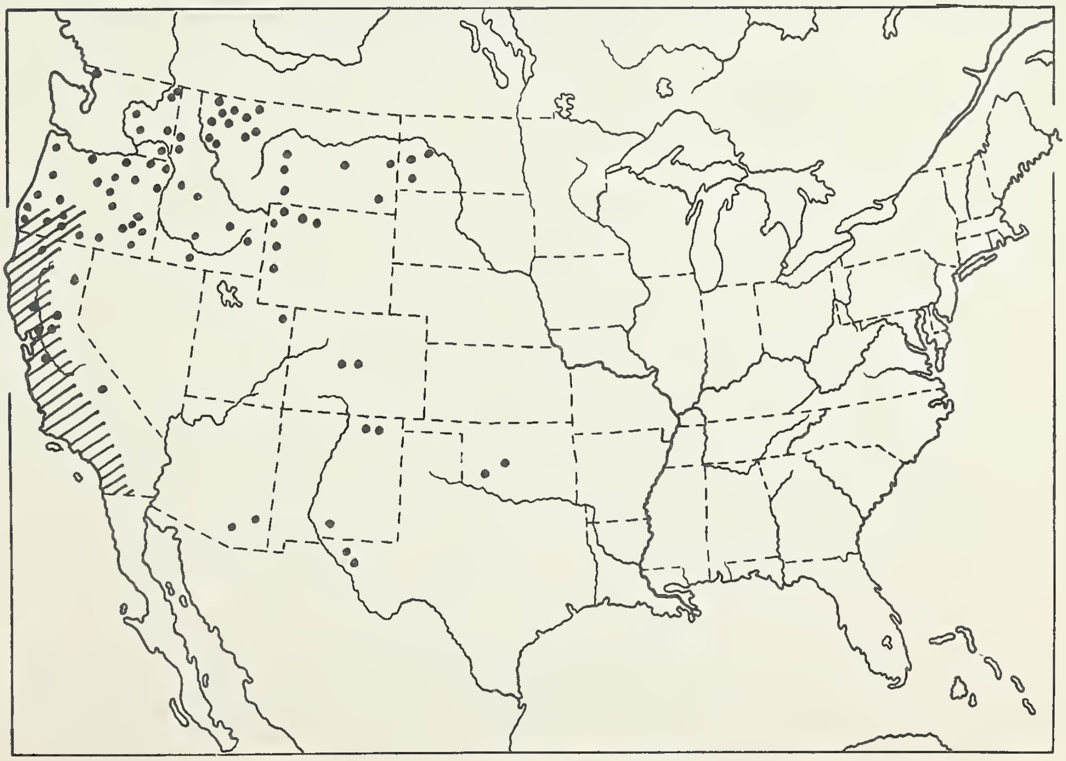


The Distribution of Dermacentor andersoni Stiles (line) and Dermacentor occidentalis Marx (stippled).



The western distribution of Dermacentor albipictus Packard (dots) and Dermacentor variabilis Say (cross hatch).

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# DETECTION OF ANAPLASMA MARGINALE BY MEANS OF FLUORESCEIN LABELED ANTIBODY
(Abstract)

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The present report describes the use of fluorescein-labeled anti-anaplasma globulin as a means of detecting Anaplasma marginale in the blood of cattle suspected of harboring the agent. In addition, the inhibitory effect of non-labeled homologous antibody upon the reaction will be described, indicating that the method might offer important advantages in the diagnosis of anaplasmosis.

Anaplasma bodies fluoresced when infected whole blood films were exposed to labeled antibody. The anaplasma, which occupied a marginal position with reference to the red blood cells, appeared as brilliant, yellow-green, very sharply defined round bodies. In contrast, the erythrocytes were clearly seen as grayish-green background structures. Anaplasma did not fluoresce following exposure of similar blood films to labeled normal globulin. Experiments with washed infected erythrocytes revealed no advantage over staining of whole blood.

In another trial, blood films were prepared from whole blood samples containing approximately 20 percent infected erythrocytes as determined by the Giemsa staining method. Lysis of the erythrocytes was accompanied by treating the films with distilled water for one minute. When the preparations were treated with homologous labeled antibody, numerous anaplasma were demonstrated, exceeding in number the above reading.

The specificity of the fluorescence reaction was investigated by setting up inhibition tests as recommended by Coons and Kaplan (1). Films by infected whole blood exposed to unlabeled anaplasma antiserum were washed and treated with labeled homologous antibody. Inhibition of the fluorescence resulted. Application of a modified inhibition test as described by Goldman (2) was also studied. Films of anaplasma-parasitized erythrocytes were exposed for 30 minutes to a mixture consisting of one drop of labeled and unlabeled anaplasma antiserum, respectively. Considerable inhibition of fluorescence was observed. Specificity of the reaction was further ascertained by exposing similar preparations to mixtures of labeled antibody and normal serum. No inhibition occurred in this system.

Finally, whole blood films prepared from a carrier animal, in which no anaplasma was demonstrated by Giemsa stain, were treated with labeled antibody. Several bodies resembling anaplasma became fluorescent in such preparations.

## SUMMARY

A globulin fraction was separated from sera of calves experimentally infected with Anaplasma marginale and conjugated to fluorescein. Alcohol-fixed organisms present in the infected blood films became fluorescent when exposed to the conjugated globulin. The immunological specificity of the staining demonstrated was ascertained by employment of suitable controls. The ability of an unlabeled immune serum to block the fluorescence reaction offers a means of testing unknown sera for the presence of the antibody.


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# ANTIGENS AND THEIR PRODUCTION

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The Oklahoma station has been making antigen for its own experimental use since 1953. All of the antigen that has been made has been used here at the experiment station in the complement fixation test, or in experiments in producing antibodies in experimental animals, or in attempts at immunization.

The following is a brief summary of our original method of preparing antigen:

Infect a splenectomized calf with anaplasmosis. At the height of infection, draw out 1500 cc of blood and inject intravenously into another splenectomized calf. At the height of disease, repeat the procedure using a third splenectomized animal, usually a two-year-old steer. This animal is then bled out at the time when the number of parasites per cubic millimeter reaches a maximum as determined by the following equation: The number of red cells per 1 cmm, by percentage of red cells parasitized equal the number of parasites per 1 cmm. The blood is collected in sterile flasks using 20% sodium citrate as an anti-coagulant. The blood is then centrifuged and the serum discarded. The packed cells are then washed four times with saline until all serum and citrate is removed. Ten volumes of distilled water is then added to the packed cells. This suspension is agitated vigorously and refrigerated over night. The suspension is then filtered through several layers of cheese cloth, then centrifuged in 50 ml. nitro cellulose tubes at 5000 R.P.M. for thirty minutes after which time the supernatant is drawn off, care being taken not to remove too much sediment at one time. This procedure is repeated until the supernatant is clear and a small button of packed sediment is visible on the bottom of the tube. This small is then re-suspended in a small volume of distilled water and lyophilized for 18 hours in three ml. aliquots.

This procedure, it is readily seen, is a long, time-consuming job unless one has access to a number of centrifuges.

In 1955, an electric Sharples centrifuge was obtained. Since then, the last step of the operation has been simplified in that we simply run the lysed cells through the Sharples at a speed of approximately 29,000 R.P.M. at the rate of one liter per hour. Care should be taken to first remove all serum and citrate before the cells are lysed. The concentrated antigen is then cleaned

off the inside of the clarifying bowl and lyophilized. This method so far has produced a good, usable antigen.

At the time we first started making antigen, we had no deep freeze in which to preserve the antigen after it was made. In time, the antigen tended to become anti-complementary if not lyophilized. The antigen was always tested to see if it was of desirable strength, and not anti-complementary, before it was lyophilized.

The question has been raised as to whether distilled water lysed cell antigen loses any of its potentcy during lyophilization. We have never run any qualitative tests to determine this. In our experience, we have found that after the antigen has been lyophilized and titrated, a given weight of that particular antigen has a constant titre. Whether this titre is of a lower value than before lyophilization has not been determined at this station.

In trying to cut down on the time consumed, we tried cutting down on the number of times the cells were washed with saline. This gave us an antigen that was anti-complementary. This is possibly due to the combining of antigen from the lysed cells with some antibody present in serum which had not been completely removed. So we feel that this is an important step.

Another obstacle encountered was high bacterial contamination of the antigen if it was made during warm weather, unless all precautions were used to keep down the contamination. Caution should also be exercised when large doses of infective blood was used to inoculate splenectomized calves for antigen production, as some calves seem to tolerate larger doses of inoculum than do others.

I have described here very briefly the methods we use for antigen production. While making these antigens, we have tried several variations, with varying results in the type of antigens produced. From the differences we have encountered by using these small variations, I would like to make these comments. I strongly recommend the need for a centrally produced, uniform standard antigen which can be obtained by all diagnostic and research laboratories throughout the country. I think it especially important that all diagnostic laboratories use the same standard antigen.

Enough antigen should be made and pooled for a several year supply. This would then be titrated to be used at a standarized dilution. Even with a standardized uniform antigen, the test is complicated enough that there are going to be some variations in the test in different laboratories. However, it would be a big step towards having a test that could be uniformly interpretated if the antigen was uniformly standard and titrated.

# STANDARDIZATION OF ANTIGENS
(Abstract)

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There is a need for the development and recognition of an official diagnostic test for anaplasmosis to be used for incidence surveys, field control programs, import, export, and interstate movement, as well as for better comparative studies by research workers.

The past difficulties encountered in the brucellosis antigen production and testing program illustrate the need for early recognition, study, discussion, and agreement concerning a method for standardization of the complement-fixation test.

The anaplasmosis-test standardization should include a uniformity of testing materials, testing methods, and testing interpretation to obtain similar results by different laboratories.

It is recommended that the antigen amboceptor, complement, and control sera be produced, standardized, packed, and distrubuted from one central control agency to assure uniformity of testing materials.

# THE TECHNIQUE OF THE COMPLEMENT FIXATION TEST FOR ANAPLASMOSIS

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The complement fixation test for anaplasmosis as applied at the Montana Veterinary Research Laboratory is essentially the same as the test used by the Animal Disease and Parasite Research Branch of the Agricultural Research Service, except that the quantities of reagents are reduced.

Reagents. -The antigen used has been supplied by the Animal Disease and Parasite Research Branch.

The complement used in pooled serum from 15 to 20 guinea pigs, stored at minus 35°C.

For the blood cell suspension, normal sheep blood is drawn aseptically into an equal quantity of sterile Alsever's solution, in which it can be stored without deterioration for 30 days at 4°C. A 3% suspension of the washed cells is used.

The amboceptor used is obtained from the Difco Laboratories.

The diluting fluid used is a Veronal-bicarbonate buffered physiological salt solution.

Equipment. - The tubes used are 15 X 85 mm. culture tubes, as used for the Kollmer test.

The racks are the Army Medical type, stainless steel, with holes of a size to take the 15 mm. tubes.

An automatic pipette is used for adding the saline, complement and the mixture of cells and amboceptor.

Methods. - The total quantity of all reagents is 3 ml. as compared with the 5 ml. used at Beltsville. This quantity in the Killmer tube is easy to mix and to read. The amount of serum tested is 0.1 ml., which is added to 1 ml. of buffered saline in the front row and to 1.5 ml. of saline in the back row. The complement, antigen and amboceptor are prepared so that 0.5 ml. of each reagent contains 2 units. The antigen and complement are added in 0.5

*Paper read by E. A. Tunnicliff.

ml. amounts. The ambocepter is mixed with the 3% blood cell suspension in equal amounts, and 1 ml. of the mixture added.

With this set-up, the result of the test of 0.1 ml. serum should be comparable to the results on 0.2 ml. serum in the A.R.S. test, the only difference being that there is slightly greater dilution with saline in our test, because our total quantity is 3 ml. as compared with 5 ml., while our serum quantity is exactly one half of the quantity used by the A.R.S. That fact, however, does not alter the relative amounts of the active reagents.

In titration of the serum, the A.R.S. calls the standard set-up a 1-5 dilution, because 0.2 ml. of serum is added to 1 ml. of saline. On that basis our set-up is a 1-10 dilution, because we start with 0.1 ml. serum in 1 ml. saline. Actually there is no difference in serum dilution in relation to the other active reagents. Therefore, the designation of the basic test as run by the A.R.S. as representing a 1-5 dilution leads to confusion. To clarify this point we suggest that the basic test be reported as the test of undiluted serum.

We are using a 3% cell suspension instead of 2%, because we think that the heavier suspension facilitates the reading, especially if the reading is made immediately on removal from the water-bath. It would seem possible that the heavier cell suspension would affect the result because the serum antibodies would have to combine with more complement to prevent hemolysis. However, in practice we have not been able to detect any difference in our readings.

In our routine method the test is not read immediately at the end of the final incubation. The racks are removed from the water-bath to a cart and wheeled into the refrigerator where they stand over night to be read the next morning. The cells have then settled completely, and a more precise reading can be made, based on the color or absence of color in the supernate, and the amount of cells in the sediment. There is no deterioration as the result of storage over night at about $4^{\circ}C$.

Comparison of the results on 1,600 samples, including some titrations of reacting serums, run by the A.R.S. and by the Montana laboratory, indicates that there is in general good agreement, but that there are probably differences in reading and recording the degree of fixation of complement. Our technician records a 4-plus reading only when the supernate is perfectly colorless, and a straight negative only when there are no un-hemolyzed cells at the bottom of the tube. In this connection, we have found that the serums from completely clean herds produce clear negative results.

If the complement fixation test is to be used in the control of anaplasmosis, it is evident that standardized methods are needed, including the use of standardized control serums for titration of antigen.

# PROCEDURES FOR THE COMPLEMENT FIXATION TEST

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The details in complement-fixation (CF) procedures are varied among different laboratories; however, the principles underlying these procedures are similar. The c.f. procedure reflects an estimate of the relative reactivity of sera and antigen with complement. The accuracy of any complement-fixing system, in terms of diagnostic application, depends primarily on (1) its inherent specificity, (2) the validity of the interpretation of results, and (3) the strict adherence to uniform techniques.

At an earlier conference on anaplasmosis, a member of the U.S. Department of Agriculture presented a c.f. procedure for the diagnosis of bovine anaplasmosis which essentially followed the U.S. Army Medical Corps' method as developed by Kent, Bukantz, and Rein. The results obtained with this method were compared with those obtained by the older method used for many years by the former Bureau of Animal Industry for the diagnosis of dourine and glanders. As there appeared to be very slight disparity of results between the two methods, and as the latter procedure was more adaptable to the routine screening of large numbers of sera, most of our testing has been carried out with the original Bureau method.

A uniform method for preserving sera is essential, as many of the samples we receive are collected and processed in the field and are shipped by mail to the laboratory. After considerable trial and error, the preservative found to be most reliable was phenol at a final concentration of 0.5%. The effect of phenol is to preserve the sera and also to stabilize the antigen-antibody reaction. We have observed that nonphenolized sera collected from proved infected animals have, on some occasions, failed to produce positive reactions, whereas duplicate phenolized samples gave the expected positive reactions. Sera collected from many proved noninfected cattle have not been altered in reaction by the addition of 5% phenol sufficient to make a final concentration of 0.5%. An excess of phenol in the magnitude of twice the recommended strength borders on the dangerous side in terms of possible nonspecific reactions.

Many laboratories experience difficulty from anticomplementary reactions with bovine sera in c.f. work when applying techniques used to test human sera. We rarely observe anticomplementary properties in bovine sera unless gross decomposition of the sample has occurred. The sera are diluted 1:5 for the screening test in normal physiological saline and inactivated at 58°C. for 35 minutes. Inactivation temperatures above 60°C. for this

period rapidly destroy the anaplasmosis c.f. antibody. The diluted sera is then removed from the waterbath and held at room temperature for at 1 hour. Fewer difficulties with false-positive reactions with samples collected from splenectomized calves showing Hemobartonella and/or Eperythrozoon infections have been observed by following this procedure, in contrast to the previous method of adding antigen as soon as the contents of the tubes reached room temperatures.

The antigen used in the c.f. test is taken from a pool of a number of individual antigens produced separately from many animals. The pooling of antigens is essential in order to have a large quantity of antigen which is uniform in properties. Each component antigen of the pool is tested and found satisfactory for use before the final pool is made. The completed pool of antigen is standardized by titration with a reference positive and negative serum. The majority of anaplasmosis antigens are slightly anticomplementary when not in the presence of normal bovine serum. The ability of normal bovine serum to overcome this anticomplementary property of the antigen makes possible a satisfactory working range between the antigenic and anticomplementary units, the latter being measured in terms of amount of antigen necessary to show anticomplementary properties in the presence of a 1:5 dilution of normal bovine serum. The amount of anaplasmosis antibody in the positive sera which is used for antigen titration determines the quantity of antigen which becomes the antigentic unit. We use a pool of positive reference sera which represents the antibody from five carrier cases of anaplasmosis. This pool has been used as our standard positive serum for antigen titration for several years, and the sensitivity of our testing reflects the corresponding antibody level of this serum. Sera collected from acute or subacute cases of anaplasmosis should not be used to standardize antigens, because the antigenic unit would then be insufficient to produce a positive reaction in the case of most sera from carrier cases which have a much lower level of c.f. antibody.

Titrations of positive sera to determine the end point of relative antibody strength are performed with a constant amount of antigen (1.5 units). The sera are diluted 1:5 and inactivated as when first tested. Then serial dilutions are continued in a diluent consisting of 5% of the reference normal bovine serum in physiological saline. The presence of the constant amount of normal bovine serum serves to counteract the increasing anticomplementary reaction of the antigen, which becomes more and more evident as the test serum becomes more dilute.

Complement is the most labile component of the system. The complement should be prepared from healthy guinea pigs which have been on a daily diet containing fresh green feed.

We have had unsatisfactory results from using some lots of commercially prepared lyophilized complement. Our practice is to collect a large pool of complement about once every 6 months in order to have a uniform

source of complement available throughout the year. Difficulties have occurred in obtaining potent complement in the late winter months.

We have attempted to approach a degree of standardization with those laboratories using antigen made at Beltsville by supplying them with the same positive and negative sera which we use to titrate antigen. Further, we have supplied each cooperating laboratory with a set of sera collected from known infected and noninfected animals in order that they can check their test results on sera of known status. In some cases we have also sent out portions of the same lot of complement being used in our laboratory in order to establish greater uniformity in testing reagent. We consider such an effort in standardization to be essential for uniform results; however, the anaplasmosis research group cannot further expand this service and also continue our primary mission on anaplasmosis research. Laboratories doing diagnostic testing should periodically receive a set of coded reference sera to determine the accuracy of their testing procedure. Such a check test on samples collected from animals of known status would help to assure the continuing accuracy of the different groups conducting the test.

As the next logical step toward greater standardization of the c.f. test for anaplasmosis, it would seem advisable to consider means whereby uniform and standardized reagents could be produced for use by all laboratories conducting tests. Antigen should be produced in large pools in one laboratory in order that all diagnostic testing can be done with a common supply of this reagent. Complement also should be produced in large quantities for common use in the various laboratories. Additional research studies are needed to provide more information on the significance of weak c.f. reactions and on the causes contributing to false-positive reactions. These difficulties with the c.f. test for anaplasmosis are intimately related to the procedural problems in standardization.

# STANDARDIZATION OF THE COMPLEMENT-FIXATION TEST Procedure

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Along with our routine anaplasmosis research we have conducted a complement-fixation test survey of some 11,000 animals. From our experience here at Kansas State we have learned that clean glassware is essential in conducting the complement-fixation test. Our test tubes are examined and sorted so that all tubes have a wall of equal thickness. The tubes are thoroughly washed and then rinsed in distilled water.

Complement is titrated each day the test is to be run. The complement is stored in a dry ice chest and we make no effort to refreeze complement once it is allowed to thaw. No doubt, our complement has probably been the major source of error in conducting the complement-fixation test at this station.

We have used both the A.R.S. antigen and an antigen made here at our laboratory. Once the titre of an antigen is established, it changes very little. Unused antigen should be refrozen, and it may be used in future tests.

Hot air incubation for both the antigen-complement and the hemolytic system has proven satisfactory for the test. We have been able to produce our own amboceptor and have been fortunate in having titres as high as 1:7500.

Five cubic centimeter total volume test has been the general procedure. The test is read immediately upon removal from the incubator. Both known positive and negative sera are employed in the test and used as a guide in reading the test results on unknown samples. Hemolysed samples have presented a problem in the survey that we conducted. Because they have proven unsatisfactory for the complement-fixation test, they are now discarded upon arrival. In any eradication program, the need of a good serum sample should be explained to those responsible for drawing the blood.

# INTERPRETATION OF THE COMPLEMENT FIXATION TEST
(Abstract)

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Data is presented to illustrate the c. f. reactions obtained from known infected and non-infected cattle. A record of the accuracy of the c. f. test based on the interpretations applied to regular bleedings of experimental animals is reviewed. The validity of the basis used to interpret c. f. reactions of field sera has been investigated by making animal inoculation tests from a number of field carrier cases. A small percentage of false-positive reactions are known to occur; however, the test is very efficient in detecting carrier cases of anaplasmosis.

# INTERPRETATION OF THE COMPLEMENT FIXATION TEST

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A clear cut interpretation of the value of the complement fixation test for the eradication of anaplasmosis cannot be obtained from Montana data. However, the presentation of these figures may contribute a better understanding of the problem. We believe that we are faced with a not too active vector and secondly, that the organism is not as virulent as in some of the sections of the country. For the most part, our anaplasmosis is centered in the north-central part of the state and the other heavy focus of infection is in southeast Montana.

The Brewster cattle are located in southeastern Montana near Birney. This herd has been under observation for some years and extensive complement fixation testing was done in 1955 and 1956. The anaplasma organism doesn't seem to be too virulent as judged by death loss among the animals. It will be noted in Table 1, there were 1611 cattle tested in 1955 with 25% reacting and 42% being suspects and but 32% negative.

The same table gives the sex and age distribution of reactors, among the 25% or 403 reactors. There were 28% cows, 15% mature bulls, 13% yearling heifers, 15% yearling bulls, 20% steers and 6% calves.

As shown in Table 2, 386 of the 1-10 reactors (25%) given in Table I were titrated out. There were 14% of the 1-10 reactors that gave 1-80 or higher fixation.

TABLE I

BREWSTER TEST 1955

| | |
|---|---|
| Total cattle tested | 1611 |
| Reactor (1-10) | 25% |
| Suspect | 42% |
| Negative | 32% |
| DISTRIBUTION OF ABOVE (25%) Reactors | |
| Cows | 28% |
| Mature bulls | 15% |
| Yearling heifers | 13% |
| Yearling bulls | 15% |
| Steers | 20% |
| Calves | 6% |

TABLE II
SERUM TITRATIONS,
BREWSTER REACTORS

| Titre | No. of Head |
|---|---|
| 1-10 | 103 |
| 1-20 | 134 |
| 1-40 | 92 |
| 1-80 | 33) |
| 1-160 | 16) 14% |
| 1-320 | 7) |
| 1-640 | 0) |
| 1-1380 | 1) |
| Total: | 386 |

The following year in November 1956, 440 calves were tested. (See Table 3). Of these 70% were non-reactors, 10% suspicious and 19% were reactors.

While there is quite a significant difference in the percentage of reactor calves in the two years, still this difference should be disregarded because of the small number of calves (24) tested in 1955.

TABLE III

BREWSTER CALVES,
November 1956

| | |
|---|---|
| Total tested | 440 |
| Reactors (1-10) | 19% |
| Suspicious | 10% |
| Negative | 70% |

TABLE IV

CHIPPEWA CREE CATTLE

| | 1955 | 1956 |
|---|---|---|
| Total tested | 1607 | 1345 |
| Reactor | 11% | 8% |
| Suspicious | 14% | 18% |
| Negative | 74% | 72% |

Titrations, 1956 Tests

| | |
|---|---|
| Titre 1-10 | 62 |
| 1-20 | 31 |
| 1-40 | 7 |
| 1-80 | 3 |
| Total: | 103 |

Table 4 shows the results of the 1955 and 1956 tests of the Chippewa Cree Tribe cattle. These cattle are owned by the Chippewa Cree Indians and are run together as a common herd. The reservation is located near Box Elder, in the northcentral part of the state. You will note the total number of reactors for the year 1955-56 were 11 and 8% respectively. The suspects ran 14% and 18% leaving negative groups of approximately 75% of the cattle. The percentage difference in reactors for the two years isn't significantly great. We have therefore concluded that the spread of the disease wasn't rapid; hence, probably a relatively inactive vector.

The lower half of Table 4 shows the 1-10 titre of the blood from 103 animals bled in 1956. In this group of 103 reactors at the 1-10 dilution there were but 3 complete titres at 1-80.

Data collected by Dr. T. O. Roby from anaplasmosis known reacting, suspects and non-reactor cattle has shown the accuracy of the test to be 98%.

The 1950-1956 records of the Montana Veterinary Research Laboratory show 2389 cattle from 11 herds varying in size from 35 to 1199 head to be completely negative except for two suspects in the largest herd. See Table 5.

TABLE V

HERDS WITH NO REACTORS

| Year | Name of Owner | Total | Negative | Suspects | Reactors |
|---|---|---|---|---|---|
| 1950 | Staunton | 300 | 300 | 0 | 0 |
| 1951 | Tamblyn | 35 | 35 | 0 | 0 |
| | Osness | 37 | 37 | 0 | 0 |
| | OXO | 144 | 144 | 0 | 0 |
| | Haryna | 50 | 50 | 0 | 0 |
| | Rugg | 64 | 64 | 0 | 0 |
| | Sannaker | 59 | 59 | 0 | 0 |
| 1955 | Spencer | 240 | 240 | 0 | 0 |
| | Stanley | 161 | 161 | 0 | 0 |
| | Fulmer | 100 | 100 | 0 | 0 |
| 1956 | McChesney | 1199 | 1197 | 2 | 0 |
| Total: | | 2389 | 2387 | 2 | 0 |

The very fact that 11 herds, presumably not infected would test completely negative with only the two serums showing in complete fixation indicates very strongly to those of us not having access to data such as Dr. Roby's that the test certainly is very specific. These negative tests give the fieldmen tremendous confidence in the test.

# AN INTERFERENCE PHENOMENON OBSERVED IN ANAPLASMOSIS RESEARCH

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An interference phenomenon between anaplasmosis and eperythrozoonosis (reciprocal) in splenectomized calves was recognized December, 1953. This report includes current research and a review of the anaplasmosis research records at the Louisiana Experiment Station dating from 1943. A total of 197 calves were used during this research.

Bovine eperythrozoonosis and anaplasmosis are two separate and distinct disease entities of splenectomized cattle. It is evident that the former is widespread in Louisiana cattle but either does not cause clinical signs or perhaps such signs have occurred but have not been properly interpreted in non-splenectomized cattle. On different occasions eperythrozoonosis has occurred spontaneously in baby calves after splenectomy. Anaplasmosis has been a problem to the cattle industry for many years.

The average anaplasmosis incubation period, when there is no interference from eperythrozoonosis, is 14 days in the splenectomized calf given 5 ml. of anaplasmosis infective blood. Whichever of the two infections is dominant in the carrier animal will be established first in the recipient anaplasmosis-eperythrozoonosis-susceptible calf. If the mild form of eperythrozoonosis is established first, the anaplasmosis incubation period may be prolonged 19 to 61 days. However, if eperythrozoonosis is acute and the splenectomized calf survives, anaplasmosis may be blocked out indefinitely unless a second or third inoculation of anaplasmosis infective blood is given. If acute anaplasmosis is the initial infection and the splenectomized calf survives, the anaplasmosis is replaced by eperythrozoonosis. In other splenectomized calves, microscopic studies show that the two diseases exist simultaneously. The mild, acute or peracute form of eperythrozoonosis can be present in some animals even though eperythrozoa can not be demonstrated microscopically.

There are 23 animals in the dairy herd of the Louisiana Agricultural Experiment Station at Jeanerette, Louisiana. Two ml. of pooled blood from 4 of these cows was given splenectomized calf 70584 intravenously February 19, 1957. Twenty-three days later this calf was given 20 cc. pooled blood from the same cows. At the same times another splenectomized calf 70581 was given the same amounts of pooled blood from 5 different cows. At the time of the second inoculation, 70581 was showing clinical signs of eperythrozoonosis and died with the peracute form of the disease 30 hours

following the second inoculation. Thirty-eight, 44, 56, 66 and 88 days following the first inoculation, calf 60584 received pooled blood from the above mentioned 9 cows in respective amounts of 50 ml. and 50 ml. intravenously; 360, 380 and 340 ml. subcutaneously.

Two days (April 18) after calf 70584 was given 360 ml. of pooled blood from the 9 cows, 25 ml. of his blood was administered splenectomized calf 70587 intravenously. This calf showed clinical and microscopic signs of eperythrozoonosis 4 days later. The peracute form of the disease developed and 70587 died 15 days after inoculation.

Calf 70587 showed clinical and microscopic signs of eperythrozoonosis 3 days after receiving 380 ml. of pooled blood from the 9 cows. These signs persisted for 6 days; the mild form of the disease was evident.

Six days after 70584 received 340 cc. of blood from the 9 cows anaplasma bodies appeared in his red blood cells. Anaplasmosis then developed in this calf.

Eperythrozoonosis interference of anaplasmosis infection is possibly responsible for the failure of splenectomized calves to develop anaplasmosis for extended periods of time after receiving 5 to 380 ml. of anaplasmosis-infective blood rather than a decreased virulence of the causative agent.*

Since the causative agent of eperythrozoonosis attacks the same cell enzyme system or systems that the etiological agent of anaplasmosis does, there are two possibilities in connection with the anaplasmosis C.F. test:

1. Eperythrozoonosis could interfere with the development of the anaplasmosis causative agent, thereby decreasing or terminating the amount of C. F. antibody production.

2. The causative agents of eperythrozoonosis and anaplasmosis could possess related antigenic substances which are capable of producing similar C. F. antibodies. In this case the serum of a bovine carrier of eperythrozoonosis, but free of anaplasmosis would give a positive reaction to the anaplasmosis C. F. test.

REFERENCE

* Gates, D. M., Madden, P. A., Martin, W. H., and Roby, T. O.: The Infectivity of Blood from Anaplasma-Infected Cattle as Shown by Calf Inoculation. Am. J. Vet. Res., 18 (1957): 257-260.

X ANAPLASMOSIS IN SPECIES OTHER THAN CATTLE +

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The subject assigned to me this afternoon is "Anaplasmosis in Species Other Than Cattle." I will discuss only the species found in sheep at this station several years ago.

This species was encountered during an outbreak of Bluetongue in one of our larger bands of sheep in the western part of Kansas. In addition to Bluetongue being present, the speaker was of the opinion that some other disease condition was also present in this particular outbreak. This assumption was based upon the weakness and anemia that apparently were present. Blood which was citrated was brought back to the laboratory and inoculated into two yearling lambs. During subsequent routine blood examinations of these lambs, marginal bodies were observed identical in appearance with Anaplasma marginale. In addition, Eperythrozoon ovis was observed in some of the cells. Serum collected from these two lambs gave complete fixation of complement when combined with antigen prepared from Anaplasma marginale.

The finding of these anti-plasma-like bodies in sheep created particular interest, because Anaplasma marginale had only been reported from field cases in cattle and deer in this country. Experimental evidence indicates that Anaplasma marginale will survive only a short time following experimental infection in sheep. This organism, of course, is found naturally in sheep in South Africa.

Further studies were made to determine the nature of these bodies observed in the sheep and their relation to bovine anaplasmosis. Transmission was readily accomplished to other sheep by injection of 5 cc of citrate of blood. The anaplasma-like bodies appeared in the peripheral blood of the inoculated animals eight to fourteen days after inoculation. A maximum infection of 1.5% of the erythrocytes was observed in these non-splenectomized animals. There was a marked reduction in the erythrocyte count of 2-7 million cells per cubic millimeter from the pre-infection values. It is difficult to determine what organism was really responsible for these reduced values, because Eperythrozoon ovis is known to be capable of producing variable degrees of anemia in experimentally inoculated sheep. The temperature was slightly elevated in most of the animals that were inoculated. There were no visible clinical symptoms in these animals. A positive reaction to the anaplasmosis complement-fixation test occurred in all sheep inoculated, beginning about the time of the microscopic appearance of the

anaplasma in the blood stream. By further studies on splenectomized lambs and medication with neoarsphenamine, the elimination was accomplished. The course of the disease in these splenectomized lambs was identical with that of bovine anaplasmosis with the number of anaplasma organisms approximately doubling each day until a maximum erythrocytic infection of 19% was reached. This peak occurred 26 days after experimental inoculation. The number of anaplasma bodies was gradually reduced following this peak infection. A progressive anemia developed. Erythrocytic counts as low as 3 million cells per cubic millimeter were observed. Splenectomized calves were experimentally inoculated with blood from sheep, and they remained normal following inoculation without developing evidence of Anaplasma marginale infection. Workers in South Africa have demonstrated that cattle are non-susceptible to A. ovis infection.

This infection in sheep has not posed a problem in our state. The exact significance of this infection in sheep is not definitely known at this time, but it is quite evident that in cases of severe parasitism or other infections, this infection no doubt would play an important part as far as losses from these other diseases are concerned. Some work has been done with symbiotic infection of the Bluetongue virus but due to the fact that we have not been able to reproduce typical field cases of Bluetongue in our experimental animals, we have come to no conclusions as to the part that Anaplasma ovis may play in these other disease conditions. Goats seem to be more susceptible to Anaplasma ovis than sheep in that we have observed a greater degree of anemia and have had some mortality in these animals. The mortality in our experimental sheep has been low, but we have lost a number of the animals during hot weather because of the anemia resulting in anoxia.

# ANAPLASMOSIS IN SHEEP AND GOATS
(Transmission Studies and C-F Reaction)

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The transmission of Anaplasma ovis to splenectomized calves was attempted with infective blood from sheep and goats. Defibrinated and citrated whole blood was used in these experiments in addition to direct intravenous inoculation of blood without anticoagulants. Quantities injected varied from 10 to 30 cc and contained from 1 to 12% anaplasma erythrocytic infection. All transmission attempts were negative in 12 calves inoculated. Five cc of blood from the same source produced anaplasmosis in sheep and goats. An attempt to recover A. ovis from the blood of calves two weeks after receiving heavily infected blood gave negative results, indicating that the organism had not survived in the bovine. All inoculated calves were later proved susceptible to acute A. marginale infection.

In the complement-fixation test a cross reaction was noted between A. ovis and A. marginale. Antigens prepared from A. marginale produced positive reactions with infected sheep sera, and conversely goat antigens prepared from A. ovis produced positive reactions with infected cattle sera. End-point titers in diluted sera were similar with either antigen.

Positive C-F reactions in sheep and goats began about the time of the appearance of anaplasma bodies. Titers were initially low, but increased as the disease progressed. End-point titrations reached a maximum of 1:40 or 1:80 and declined as the carrier state was reached. These highest end-point readings were considerably less than the 1:320 or 1:640 end-points obtained in bovine infections.

In A. ovis carrier infection of sheep and goats the C-F test has not been used with accuracy. Known carriers have given reactions varying from negative and suspicious, to positive at various times. In some carriers anaplasma C-F reactions were negative even though microscopic anaplasma bodies were present in the blood. It is concluded, therefore, that the C-F test is of value in confirming field diagnosis of acute cases, but not reliable in determining carrier status.

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# THE METABOLISM OF RADIOACTIVE IRON IN ANAPLASMOSIS
(Abstract)

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As part of a program to obtain additional information concerning the nature of the anemia in anaplasmosis, the rate of clearance of iron from the plasma and the rate of its incorporation into the red blood cells have been determined prior to and during anaplasmosis in 7 splenectomized calves.

$Fe_{59}$ supplied as ferric chloride was used to determine the rate of clearance of the iron from the plasma and the rate of uptake by the red cells. The rate of clearance from the plasma after intravenous injection of the iron and the rate of uptake by the red cells together with the plasma volume and frequent determinations of the hematocrit, the hemoglobin, the plasma iron and the body weight made it possible to determine the following data -

(1) the mgm. of plasma iron turned over daily
(2) the rate of incorporation of the iron (mgm/day) into the red blood cells.
(3) the rate at which the iron is released from the red cells (mgm/day)
(4) the life span of the red blood cells.

Iron transport studies were performed in the injected calves when 0.1 - 1.0% of the red blood cells were injected with anaplasma bodies. Existing data suggest that in the injected calves the plasma iron turnover rate is increased, the life span of the red blood cell is reduced and that hematopoiesis as indicated by the rate of incorporation of iron into the red blood cells is unchanged or depressed.

# OBSERVATIONS ON THE MORPHOLOGY OF ANAPLASMA MARGINALE WITH REFERENCE TO PROJECTIONS OR TAILS

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## Source and Description of Calves

Six grade yearlings of predominantly Hereford breeding were purchased locally and splenectomized several weeks previous to inoculation.

Calves were numbered in consecutive order, divided in groups of 2, and each group was inoculated approximately 20 days apart.

## Source of Inoculum

A normal Hereford yearling which several months previously had gone through a rather severe attack of anaplasmosis was used as the source of carrier blood. This animal had been inoculated with blood from a recent virulent field infection of anaplasmosis.

## Procedure

Two splenectomized calves were each given 5 cc. of fresh whole blood subcutaneously at the same time from the carrier animal and temperatures were taken daily thereafter. Daily blood smears and hematocrit readings were made from the time marginal bodies were first observed until the termination of the experiment.

## Observations

### Group 1 - Calves 4618 and 4619

The incubation period (time from inoculation until bodies typical of *Anaplasma marginale* was first observed) was 21 days in each calf.

Projections or tails on the *A. marginale* bodies were seen only on the first day that these bodies appeared in the blood of each of these two calves.

### Group 2 - Calves 4620 and 4621

The incubation period was 19 days in animal No. 4620.

The projections or tails were first observed on the 24th day after inoculation with approximately 1 per cent of the cells showing typical

Anaplasma marginale. The tails were also observed on each of the 3 following days after which time tails were no longer found.

Animal 4621 died from drug treatment on the 22nd day following inoculation at which time it was beginning to show a one per cent anaplasma infection. No tails were observed.

Group 3 - Calves 4622 and 4623

The incubation period was 20 days in each calf.

The projections or tails were first observed on the 27th day after inoculation in calf No. 4622 at which time approximately 18 per cent of the RBC's showed marginal bodies. One tail was observed on the following day. There was none found afterward. This calf died 4 days later.

Tails were seen previously and also on the 28th day following inoculation in calf No. 4623 which was also the day the calf died. Approximately 30 per cent of the RBC's showed marginal bodies and the animal's hematocrit reading was 8 at the time of death.

Time from first appearance of anaplasma bodies until the calves died ranged from 9 to 15 days.

Discussion

In Dikman's[1] review of the literature concerning the morphology of anaplasmosis he quotes several authors as follows:

"Gomes de Faria[2] —— . Besides these regular rod forms, there are at times comma like forms with a dense spherical portion with a filamentous tail which at times appears double. These forms correspond to those seen by Quevado[3] and Descaseaux[4]. Quevado, in one of his articles considered these forms as being due to the throwing out of fine prolongations in the shape of flagella and Descaseaux mentions their occurence in organ smears of animals dead of anaplasmosis.

We observed in one slide, the bodies described by Gomes de Faria. However, none of Dikman's photomicrographs in his article showed what we are attempting to describe in this paper.

Lotze and Yiengst[5] in their able presentation on Studies on the Nature of Anaplasmosis may have described what we are reporting although their photomicrographs make it difficult to definitely establish this fact. Also, we find it impossible to say that they are extra-erythrocytic. They definitely appear to be attached to the marginal body in the cell. This observation has been confirmed by several microscopists.

The bodies appear to be within the ordinary size and location of typical _A. marginale_ (.2 to .5 micron). The body stains a deep purple or magenta with Giemsa stain and the tail portion usually stains a light pink and extends outside of the red cell. However, if the slides are stained with alkaline water for the Giemsa stain solution, the tails take a basophilic stain and are quite apparent. The tails vary in length but their length may exceed the width of a large anaplasma body (.6 to .9 micron).

These tails have been observed on a few occasions in non-splenectomized calves.

## Summary and Conclusion

Observations were made on 6 splenectomized calves that were inoculated with blood from an animal known to be carrying anaplasmosis. Projections or tails extending from typical anaplasma bodies were observed at different times in 5 cases. Similar tails have since been observed in the blood of non-splenectomized calves that were infected with anaplasmosis.

It is probable that this represents one stage in the normal development of _Anaplasma marginale_ in the blood of cattle.

This phenomena has only been observed with this strain of _Anaplasma marginale_.

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# CHEMOTHERAPEUTIC CONTROL OF EXPERIMENTAL BABESIAL AND ANAPLASMAL INFECTIONS IN BRAZIL

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The South American cattle disease commonly called "tristeza" has been studied by Lignère[1] in Argentina and Esquibel[2] in Brazil and methods of treatment have been developed. It is an acute or chronic infectious disease, consisting of both piroplasmosis and anaplasmosis associated with bodies in the erythrocytes referred to as *B. bigemina*, *B. argentina* and *Anaplasma marginale* which are transmitted by the same vector, the tick *Boophilus microplus*. Imported valuable cattle and their progenies are especially susceptible to attack of the haemosporidia and anaplasma, resulting in a high mortality rate unless treated properly.

The most successful solution practiced in Brazil to the problem of protection of imported cattle susceptible to both diseases consists of a premunition program in quarantine stations, during which the animals receive one subcutaneous injection of blood from an immune carrier. The animals are subsequently observed for febrile reactions and increase of parasite count which occur at 8-11 days for babesia and at 21-38 days for anaplasma. As soon as these reactions are observed, the animals are treated with parasiticides such as Acaprin, Acriflavine or Trypan blue, which restore temperature to normal without sterilizing their blood, so that the animals become immune carriers. They are finally challenged with ticks carrying babesia and anaplasma and, after they are found free of any clinical manifestations of "tristeza" for one month, they are sent out to the individual farms. However, the drugs used in the past for chemotherapeutic control of the reactions occurring during premunition, have a number of disadvantages. Some of these produce flebitis, liver damage and sometimes photo-sensibility, whereas with others, undue side effects occur, mostly caused by parasympathetic stimulation which necessitates the simultaneous use of other drugs. Still others result in discolorization of muscle tissue, so that after slaughtering the meat shows an undesirable bluish tint.

Due to the development of the broad spectrum antibiotics, the use of chlorotetracycline, oxytetracycline and tetracycline in anaplasmosis has been investigated in the United States[3-8] and it has been shown that relatively large doses of these antibiotics (50-100 mg./kg.) have resulted in complete destruction of the carrier state of anaplasmosis in cattle. Since the premunition against anaplasmosis is based on survival of some

parasites in the organism of the treated animal, it appeared promising to us to test tetracycline at relatively small and economical doses (2-5 mg./kg.) for controlling the febrile reactions often followed by more severe complications, which occur as undesirable features during premunition. Ribeiro Netto and Pereira Lima[9] have reported that small doses of terramycin (0.5-6.0 mg./kg.) can be useful during the course of premunition against bovine anaplasmosis in Brazil which further stimulated our interest in testing tetracycline for the same purpose.

The object of this study was to test the efficacy of p,p'-diguanyl-diazoamino-benzene and small doses of tetracycline hydrochloride respectively in the chemotherapeutic control of experimental babesial and anaplasmal infections as produced during premunition of susceptible cattle in Brazil.

## Methods

The study was conducted at the Sao Paulo Agua Branca quarantine station during routine premunition of imported valuable cattle and no untreated controls were available; therefore, negative results in fever control at low drug dosage levels have to be considered as controls in the described experiments.

The animals used were all pregnant imported cows, of the Holstein type. They were experimentally infected by subcutaneous injection of 5 cc. blood from an old, constantly tick infested carrier, showing positive smears for B. bigemina, argentina and A. marginale (Giemsa stain). The animals were subsequently kept in individual tick free stalls and maintained under optimal clinical conditions as regards hygiene and alimentation. Anal temperatures were taken twice daily.

Usually 21-38 days after experimental infection, the same animals showed a renewed rise of temperature due to increase of Anaplasma marginale. Outside of this manifestation of the disease, other clinical symptoms of sickness were general weakness and lack of appetitie. The criterion used for beginning the treatment with tetracycline hydrochloride was a temperature of 40°C and anaplasma counts of at least 3%. As criterion for a positive result of treatment was chosen the return of temperature to 39.5°C or less within 48 hours and no temperature rise for further 5 days. Tetracycline hydrochloride (oral grade) was employed in an aqueous solution of at least 5 concentration.

One week after reestablishing normal body temperature, all animals were challenged with Boophilus microplus carrying B. bigemina, argentina and A. marginale, and kept under observation for at least four weeks.

Three normal control animals were used to demonstrate the presence of virulent babesia and anaplasma in the ticks.

The premunition method described is routinely used in Brazilian quarantine stations for imported cattle before the animals are sent to farms in the interior of the county.

<u>Use of tetracycline in Anaplasmosis produced by pre-immunization in Brazil</u>

Table I summarizes the results obtained in the tetracycline treatment of fever reaction occurring during the premunition against anaplasmosis in the same cattle which previously were premunized against piroplasmosis and treated with Ganaseg.

Nine of the 47 cows had no febrile reaction and blood counts of anaplasma less than 3%. They were challenged with ticks at the same time as the drug-treated animals and showed complete resistance. This might indicate a relatively low virulence of the anaplasma of the carrier or a high degree of resistance of some of the animals; it can be noted that cows nos. 19 and 36 neither showed reactions to the clinical babesial infection. All of the remaining 38 cows had febrile reactions accompanied by increase in count of anaplasma 30.4 days (21-38) after receiving the injection of blood from the immune carrier.

Table I shows 100% cure in 12 cows injected i.v. with 2.5 mg./kg. tetracycline hydrochloride, whereas the same amount given i.m. resulted in cure of only five of eight animals (62.5%). In three animals, the temperature rose again to 40°C within 3-8 days after the first i.m. injection, accompanied by rise in anaplasma counts, whereupon the animals were treated again with 2.5 mg./kg. i.v. The higher dosages of 5.0 and 7.0 mg./kg. tetracycline hydrochloride administered i.m. resulted in 100% cures.

The doses of 2.5 mg./kg. tetracycline did not cause any undue reaction irrespective of the type of application, whereas the larger doses of antibiotic, given i.m., caused oedema at the site of injection. This effect might have been due to the use of higher concentrations of tetracycline hydrochloride than a 5% solution of pH 2.5 in relatively superficial intramuscular injections. At higher dosage than 2.5 mg./kg. i.m. it would be preferable to use a solution with a maximal drug concentration of 5.0% applied in different sites of the muscle.

A group of six cows was treated orally with 12.5 mg./kg. tetracycline. Of these, four showed cure (66.7%) of the febrile symptoms, whereas two had to be given 2.5 mg./kg. i.v. to normalize the temperature. Animal no. 24 was treated with 12.5 mg./kg. tetracycline orally and showed a temperature of 38.9°C 48 hours after treatment. The temperature rose again to 40.5° C within further 48 hours when it was treated with 7 mg./kg. i.m.* After this last treatment the cow had abortion, fever remained high

* When this treatment did not normalize the temperature, another application of 2.5 mg./kg. i.v. was made.

for another 48 hours and finally returned to normal. Due to this complication and insufficient information, this animal has been eliminated from the final evaluation of data.

The described data seem to indicate that 2.5 mg./kg. i.v. of tetracycline hydrochloride can serve as an effective and practical agent to control the undesirable clinical symptoms frequently occurring in Brazil during the course of premunition in susceptible imported cattle against anaplasmosis, tetracycline hydrochloride showing efficacy against anaplasma previously only observed for related compounds of this group.

1) 47 imported animals were experimentally infected with blood from a carrier containing the parasites B. bigemina, B. argentina and A. marginale, during the course of premunition. Piroplasmosis appeared at 9.1 days which was treated with Ganaseg, followed by anaplasmosis at 30.4 days. When the body temperature of the animals cured of piroplasmosis rose again due to anaplasmosis, treatment with tetracycline hydrochloride (oral grade) was commenced at various doses, comparing the intramuscular, intravenous and oral routes. Cures were judged by return to normal temperature within 48 hours.

2) 2.5 mg./kg. tetracycline hydrochloride, given intravenously, resulted in 100% cure of clinical manifestations without any undue reactions.

3) The intramuscular dose of 2.5 mg./kg. tetracycline resulted in 62.5% cure, whereas 5.0 and 7.0 mg./kg. resulted in 100% cure of the clinical manifestations of the disease.

4) 2.5 mg./kg. tetracycline hydrochloride injected intramuscularly, did not cause any undue reactions. The larger drug doses tested all produced oedema at the site of the injection under the conditions used.

5) 12.5 mg./kg. given orally cured 66.7% of the treated animals of the clinical manifestations of anaplasmosis without any apparent ill effect on the rumen.

6) Exposure test to ticks carrying B. bigemina, B. argentina, besides A. marginale, of the animals treated previously with tetracycline hydrochloride and cured against the clinical manifestations of anaplasmosis showed complete immunity against new attacks of the parasites. The virulence of the parasite infested ticks was demonstrated in three non-premunized susceptible calves.

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TABLE I
USE OF TETRACYCLINE DURING PREMUNITION AGAINST ANAPLASMOSIS

| Animal no. | Days between infection and treatment | Days between infection and return to normal temp. | Tetracycline dose mg./kg. and route | Repeated treatment dose mg./kg. and route | % cure after first treatment |
|---|---|---|---|---|---|
| 6 | 34 | 38 | | 2.5 i.v. | |
| 43 | 30 | 35 | | 2.5 i.v. | |
| 28 | 22 | 31 | | 2.5 i.v. | |
| 37 | 28 | 31 | 2.5 i.m. | - | 62.5 |
| 27 | 21 | 25 | | - | |
| 25 | 27 | 28 | | - | |
| 42 | 31 | 32 | | - | |
| 13 | 37 | 39 | | - | |
| 4 | 23 | 26 | | - | |
| 22 | 29 | 30 | | - | |
| 49 | 32 | 35 | 5.0 i.m. | - | 100 |
| 14 | 33 | 37 | | - | |
| 44 | 36 | 39 | | - | |
| 39 | 21 | 23 | | - | |
| 38 | 22 | 23 | | - | |
| 21 | 22 | 23 | | - | |
| 11 | 26 | 29 | 7.0 i.m. | - | 100 |
| 50 | 21 | 26 | | - | |
| 46 | 24 | 27 | | - | |
| 26 | 31 | 37 | | 2.5 i.v. | |
| 25 | 26 | 31 | | - | |
| 9 | 33 | 39 | | - | |
| 29 | 28 | 29 | 12.5 oral | - | 66.7 |
| 41 | 34 | 39 | | - | |
| 24* | 24 | 34 | | 7.0 i.m. 2.5 i.v. | |
| 48 | 34 | 40 | | 2.5 i.v. | |
| 18 | 38 | 40 | | - | |
| 16 | 37 | 39 | | - | |
| 5 | 35 | 38 | | - | |
| 3 | 33 | 34 | | - | |
| 10 | 37 | 39 | | - | |
| 23 | 37 | 39 | | - | |
| 8 | 31 | 33 | 2.5 i.v. | - | 100 |
| 40 | 38 | 40 | | - | |
| 12 | 36 | 39 | | - | |
| 52 | 37 | 39 | | - | |
| 7 | 35 | 39 | | - | |
| 51 | 32 | 34 | | - | |

*Excluded from evaluation due to complications and insufficient information.
Animals without febrile reaction and anaplasma counts below 3%: nos. 17, 19, 20, 34, 35, 36, 45, 47 and 53.

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